Inhibin and Related Peptides in Human Pregnancy

Sodsai Tovanabutra B.Sc., M.Sc.

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For My Family

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Declaration

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Sodsai Tovanabutra

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Abstract

The studies described in this thesis were designed to investigate inhibin and inhibin-related peptides throughout human pregnancy. The first approach was to characterize antibodies raised against the intact inhibin molecule or different peptide fragments of inhibin subunits in order to obtain suitable antibodies for radioimmunoassay and Western blot analysis. The results showed that two sheep antibodies, Y29 and Y33, which were raised against synthetic human inhibin α-(1-23)-NH_2, were specific to inhibin-like molecules in placental extracts and amniotic fluids. An antibody (Monash antibody) raised in a rabbit against 32 kDa inhibin was the most sensitive antibody for measuring inhibin in plasma and placental extracts. The development of the Western blot analysis for inhibin was successful with Y29 and R187 antibodies and another monoclonal antibody raised against human βA-(87-114)-NH_2. These three antibodies showed specificity to purified 32 kDa bovine inhibin and the antibodies to βA-subunit fragments also showed specificity to recombinant human activin A.

Inhibin levels were measured during pregnancy. There was an increase in inhibin levels in the luteal phase after the luteinizing hormone (LH) surge which reached a peak during the first two weeks of pregnancy. The levels of inhibin declined after this first peak and then rose again, reaching a second peak during 7-8 weeks of pregnancy. A second fall was observed until 30 weeks of gestation followed by a dramatic increase at approximately 36 weeks which was sustained until delivery. These results suggest that before the formation of the placenta, the corpus luteum is a potential source of inhibin. Human chorionic gonadotrophin (hCG), oestradiol (E_2) and progesterone (P_4) were also measured in order to investigate any interactions between inhibin and these hormones *in vivo*. The results showed that oestradiol and progesterone increased throughout pregnancy as did inhibin. hCG increased during early pregnancy and declined after 12 weeks and remained at this level until delivery.

Immunoactive inhibin levels in placental extracts from various stages of pregnancy were measured. The results showed that inhibin levels were very high at 7 weeks and decreased towards term. Placental hCG content was also shown to reach maximal concentrations at 7 weeks and was less in mid-term and term. These results suggest a possible transient relationship between inhibin and hCG, E_2 and P_4 during pregnancy.

Inhibin bioactivity in placental extracts was measured using a sheep pituitary cell bioassay. The results showed that the bioactivity of inhibin in placental extracts did not change throughout pregnancy whereas the immunoactivity decreased from early pregnancy towards term. Placental inhibin bioactivity was immunoneutralized in sheep pituitary cell cultures using inhibin α- and β-subunit antibodies. The results showed that only the inhibin α-subunit antibody could neutralize inhibin bioactivity in the placenta and the same antibody could be used to neutralize inhibin bioactivity in amniotic fluid.

Immunoactivity and bioactivity of inhibin was investigated in amniotic fluids during early, mid-term and term pregnancy. The results showed that inhibin levels measured by radioimmunoassay (RIA) with Y29 and R187 antibodies decreased towards term, whereas the inhibin level measured by the Monash antibody increased towards term. The level of bioactivity was different from immunoactivity; the lowest activity was observed in mid-term whereas the same level was found in early and term pregnancy. These results provide evidence for the presence of dimeric inhibin in amniotic fluid and may also suggest that different species of inhibin-related peptides exist in amniotic fluid at various stages of pregnancy.

The cellular localization of inhibin α- and βA-subunits was examined using antibodies raised against these two subunits. Syncytiotrophoblast and cytotrophoblast cells showed positive staining throughout pregnancy. The intensity of staining corresponding to each inhibin subunit varied at different stages of pregnancy with maximum α-immunoactivity during early pregnancy and maximum βA-immunoactivity at term.

To examine the expression of inhibin genes in placental tissue during pregnancy, Northern blot analysis using inhibin α–subunit cDNA and βB-subunit riboprobes was carried out. The results from this study showed expression of inhibin α- and βB-mRNAs by placental tissue throughout pregnancy. The α-mRNA showed greater expression in 7 weeks placentae when compared to mid-term and term placentae, whereas the βB-mRNA was abundant throughout pregnancy and its degree of expression increased in the later stages of pregnancy.

To investigate whether trophoblast cells actually produce inhibin subunits, the *in situ* hybidrization technique was employed to demonstrate inhibin gene expression. Using ^{35}S-labelled inhibin subunit riboprobes, it was demonstrated that all three inhibin

subunit mRNAs were expressed in both syncytiotrophoblast and cytotrophoblast cells. Inhibin α-mRNA was expressed throughout pregnancy and the degree of expression in early placenta was higher than in term. Inhibin βA-mRNA was also expressed in early pregnancy and term while inhibin βB-mRNA was only slightly expressed in early pregnancy.

CHAPTER 1

Literature review

Introduction

In 1923, the existence of inhibin was first suspected from the results of an experiment in which it was demonstrated that after damage of the germinal epithelium of the testis by radiation in the rat the anterior pituitary hypertrophied with the appearance of typical "castration cells" (Mottram & Cramer, 1923). This phenomenon led to the postulate that the radiation caused the disappearance of an internal secretion from seminal epithelium which might be responsible for the morphological changes in pituitary glands. Martins and Rocha (1931) demonstrated that an injection of bull or goat testicular extracts completely prevented these castration effects in male rats. In 1932, McCullagh reported that the administration of aqueous extracts of bull testes to castrated male rats resulted in the disappearance of castration cells in pituitary glands without increasing the weight of the accessory sex organs. In the same experiments, the solvent extracts were also shown to have effects on the accessory sex organs but not on castration cells. The water soluble factor was termed 'inhibin' and the solvent factor 'androtin' which was later elucidated as testosterone. However, there followed a long period of controversy on the existence of inhibin due to the lack of sensitive assays to monitor its activity. In the late 1960s, the development of an FSH radioimmunoassay allowed many studies to accumulate direct evidence for the existence of inhibin. FSH-suppressing activity characteristic of inhibin was detected in semen of various species, bovine seminal plasma, urine and spermatic venous blood (Fachini et al, 1963; Fachini & Ciaccolini, 1966; Lugaro et al, 1969; 1973). Moreover, rete testis fluid showed marked FSH-suppressing activity when tested using the suppression of uterine weight in hCG-injected mice as an end point (Setchell & Wallace, 1972; Setchell & Sirinathsinghji, 1972). In the same year, human seminal plasma was shown to suppress FSH levels in the blood of castrated male rats (Franchimont et al, 1972). Setchell & Jacks (1974) also demonstrated the same FSH-suppressing activity in ovine rete testis fluid. Evidence for the independent regulation of FSH and LH secretions was observed in testicular feminization patients with high LH levels but normal FSH levels (Judd et al, 1972) and in connection with congenital absence of Leydig cells (Berthezene et al, 1976). This phenomenon was also confirmed by normal levels of LH and high levels of FSH in patients with impaired sperm production (Setchell et al, 1977). In the cow, de Jong & Sharpe (1976)

demonstrated an inhibitory effect of steroid-free bovine ovarian follicular fluid (FF) on FSH, but not LH, secretion following castration. This direct evidence of inhibin in follicular fluid was supported by the observation of an increase in FSH but not LH levels at the menopausal transition (Sherman & Korenman, 1975) and the elevation of FSH in the woman with normal LH and oestradiol who had no follicular development (Boyar et al, 1977).

There has been a tremendous amount of information on many aspects of inhibin in the last decade e.g. sources, detection, estimation, isolation, purification, structural analysis, biosynthesis, secretion, distribution and physiological role. These aspects will be reviewed as follows:

1. Assays of inhibin

1.1 Bioassays

Bioassays of inhibin have been developed based on the ability of inhibin to suppress FSH secretion. The original *in vivo* bioassay was the reversed Steelman-Pohley assay using the suppression of inhibin on the synergistic effect of FSH to hCG in augmentation of ovarian weight of immature rats (Steelman & Pohley, 1953; Setchell & Wallace, 1972). Similar types of assay, using uterine or ovarian weight in mice or rats as the end points, were developed to measure inhibin activity (Setchell & Sirinathsinghji, 1972; Chari et al, 1976; Ramasharma et al, 1979). Another bioassay relied on the suppressive effect of inhibin on increasing ovarian weight in unilaterally ovariectomized mice (Sato et al, 1978). However, results from these bioassays were difficult to interpret because of the presence of FSH-binding inhibitors in gonadal fluids which made the assay non-specific (Sluss & Reichert, 1984). Thus, in combination with low precision and sensitivity, these types of assay had many limitations. Since the radioimmunoassay of FSH was developed (Midgley, 1967) an *in vivo* bioassay based on the suppression of FSH concentrations in blood as an end point of the injection of inhibin preparations has been extensively used to measure inhibin bioactivity. Several assays with the same principle were developed using both sexes of rats and mice under different conditions i.e intact, castrated and ovariectomized to detect inhibin activity in bovine, porcine, mare and human ovarian follicular fluids (de Jong and Sharpe, 1976; Welschen et al, 1977; Miller et al, 1979; Daume et al, 1978) and in rat, bovine and ovine testes extracts (Davies et al, 1978;

Keogh et al, 1976; Nandini et al, 1976; Sheath et al, 1979) and in ovine rete testis fluid (Setchell & Jacks, 1974).

The assay for the hypothalamic releasing factors using dispersed pituitary cells (Vale et al, 1972) was developed and adapted for monitoring inhibin bioactivity. At present *in vitro* bioassays using the monolayer culture of rat or sheep pituitary cells are extensively used with the FSH content of the cells (Scott et al, 1980; 1982), the FSH and LH released into the culture medium (Steinberger & Steinburger, 1976; DePaolo, et al, 1979; Tsonis et al, 1986) or the FSH and LH secreted in response to (luteinizing hormone releasing hormone (LHRH) stimulation (de Jong et al, 1979; Eddie et al, 1979) measured to monitor inhibin bioactivity. Although these bioassay systems are more specific and sensitive than the *in vivo* bioassays mentioned above, there are still some limitations to overcome. The rat monolayer culture systems are not sensitive enough to detect inhibin in circulating blood and also show specificity to follistatin (Robertson et al, 1990a) which is another type of FSH-suppressing protein. The highly sensitive ovine pituitary cell culture system developed by Tsonis et al (1986) enables the measurement of circulating inhibin. However, sheep pituitary cells are very sensitive to oestradiol and progesterone (Miller & Huang, 1985; Batra & Miller, 1986) and also testosterone (Eddie et al, 1979). Thus, it is necessary to ensure removal of all steroids from the sample by treatment with charcoal and/or immunoneutralization prior to measurement in this system (Tsonis et al, 1986). Furthermore, inhibin preparations containing some growth factors can stimulate pituitary cell differentiation and multiplication (Tsonis et al, 1987b; Tsonis et al, 1988b; Dorrington et al, 1988). These complications make the interpretation of results more complex. Nevertheless, at present the ovine monolayer pituitary cell culture is still the most sensitive assay system to determine the biological activity of inhibin-containing preparations .

1.2 Immunological measurements

Antibodies raised against intact inhibin molecules and different fragments of inhibin α- and β-subunits have been extensively used for immunoassay, immunoneutralization and immunohistochemical localization of inhibin and its related peptides in various species.

1.2.1 Quantitative immunoassays

Radioimmunoassay (RIA)

Immunoassays of inhibin have been developed using different principles. The most sensitive and widely used is radioimmunoassay which has two categories according to the type of antibody. The first type is the RIA using a polyclonal antibody against purified bovine 58 kDa or 32 kDa inhibin (McLachlan et al, 1986a; McLachlan et al, 1987a; Robertson et al, 1988a; 1988b) and purified porcine or bovine intact inhibin (Hasegawa et al, 1987; 1988). These assays have been shown to have negligible cross-reaction with other proteins in the inhibin family such as activin, transforming growth factor-β (TGF-β), and Mullerian inhibiting substance (MIS) (McLachlan et al, 1986a). Interestingly, another immunoactive inhibin species, pro-αC, which has been found in bovine and porcine follicular fluids, has been shown to cross-react more than 200% in this assay (Robertson et al, 1989; Sugino et al, 1989). In addition, preparations containing recombinant α-inhibin-immunoreactive proteins have also shown the same displacement in the assay as the standard which was pooled serum from female patients undergoing exogenous gonadotrophin therapy (Schneyer et al, 1990). However, this type of assay has been widely used to measure serum inhibin in many species under different physiological and pharmacological conditions.

Another type of assay has been developed using antibodies raised to a range of synthetic peptides representing the amino terminal region of the 20 kDa mature α-subunit of porcine 1-25 or 1-26 inhibin (Rivier et al, 1986; McNeilly et al, 1989; Schanbacher, 1988; Sharpe et al, 1988) and human 1-23, 6-30 or 1-32 inhibin (McNeilly et al, unpublished data; Sinosich et al, 1991; Groome et al, 1990; Saito et al, 1989; Mersol-Barg et al, 1990). This type of assay has been used to measure inhibin in serum, gonadal extracts and culture medium of gonadal cells or tissues from many species. Although this assay does not cross-react with other inhibin-related proteins (activin, TGF-β, MIS), it detects immunoactive α-subunits as well as dimeric inhibin. Thus, the levels of α-subunit in inhibin-containing preparations must be taken into consideration.

Enzyme-linked immunosorbant assay (ELISA)

More recently, an enzyme-linked immunosorbant assay of inhibin has been developed by a Belgian group using two antibodies directed against different α-subunit

fragments (Eliard et al, 1990). This is a direct assay for the immunoactive α-subunit but is not an ideal assay for dimeric inhibin. It has been demonstrated to be comparable with the above heterologous RIAs in sensitivity and reproducibility. Since this method is less time-consuming and labour-saving, it has been widely used in clinical research to detect inhibin levels in males, females, children and fetuses (see Proceedings of Inhibin Symposium, Clinical Investigations, Medgenix Diagnostics, 1990).

Immunoradiometric assay (IRMA)

The last quantitative immunoassay is the immunoradiometric assay using two antibodies raised against inhibin α-subunit and β-subunit fragments. This assay is theoretically highly specific for dimeric inhibin and does not cross-react with the α-subunit (Knight et al, 1991). However, at present, this assay is not sensitive enough to measure inhibin levels in human serum.

1.2.2 Immunolocalization

In the past five years, advances in immunology in combination with advanced histochemical techniques, have enabled antibodies to inhibin α-and β-subunit fragments to be used to demonstrate the localization of inhibin subunits in various tissues. The inhibin subunits are localized in different cell types of the ovary. Human and rat granulosa cells contain the inhibin α-subunit (Smith et al, 1991; Cuevas et al, 1987; Merchenthaler et al, 1987; Meunier et al, 1988a) and inhibin βA- and βB-subunits (Meunier et al, 1988a). Meunier and colleagues (1988a) also demonstrated the inhibin α-subunit in rat theca and interstitial cells. Luteal cells are also show positive staining with the inhibin α-subunit antibody (Smith et al, 1991; Cuevas et al, 1987; Merchenthaler et al, 1987; Meunier et al, 1988a). Immunolocalization of inhibin subunits in rat testicular tissue has also been investigated. Rat Sertoli cells have shown positive staining with an antibody raised against the inhibin α-subunit (Cuevas et al, 1987; Merchenthaler et al, 1987; Rivier et al, 1988; Saito et al, 1989; Shaha et al, 1989). In addition, the last two groups have also shown the localization of inhibin β-subunits in rat Sertoli cells. However, only one group has been able to detect inhibin subunits in Leydig cells (Shaha et al, 1989). The control mechanism of inhibin and FSH remains unclear but there is indirect evidence demonstrating a feedback loop between the gonads and the pituitary gland (de Jong, 1987). This study has been further confirmed by the localization of inhibin subunits in pituitary gonadotrophs (Roberts et al, 1989) and the βA-subunit has also been localized in central neural

pathways of rat brain (Sawchenko et al, 1988). Inhibin α-subunit immunoactivity has also been localized in human cytotrophoblast cells (Petraglia et al, 1987a; Merchenthaler et al, 1987) and decidua (Petraglia et al, 1990).

1.2.3 Immunoneutralization

Polyclonal antisera raised against the amino terminal of the inhibin α-subunits of bovine, porcine and human have been shown to neutralize inhibin bioactivity *in vivo* and *in vitro* (Forage et al, 1987; Findlay et al, 1989; Rivier & Vale, 1989; Saito et al, 1989; van Dijk et al, 1986; Mann et al, 1989). These antibodies have been exploited as an indirect method to monitor inhibin bioactivity *in vivo* and *in vitro*.

2. Isolation and characterization of inhibin and related peptides

Although inhibin has been shown to exist for more than half a century (Mottram & Cramer, 1923) it is only within the last decade that investigators have succeeded in its isolation and characterization after being hampered, in the intervening years, by the hydrophobic nature of the protein and the lack of specific assays with high sensitivity. The first successful isolation of inhibin from bovine follicular fluid (bFF) demonstrated a 58 kDa glycoprotein consisting of a 43 kDa subunit linked to a 15 kDa subunit by disulphide bonds (Robertson et al, 1985). Proteolytic cleavage of the 43 kDa subunit caused by the low pH conditions of the purification process gave rise to a 31 kDa peptide consisting of 20 kDa and 11 kDa subunits (Robertson et al, 1986). Similar molecular masses of inhibin in bFF were also confirmed by other investigators (Fukuda et al, 1986; Miyamoto et al, 1986; Knight et al, 1989). Moreover, a range of high molecular weight species, 65 kDa, 88 kDa, 108 kDa and 120 kDa, were identified by Miyamoto et al (1986) using monoclonal antibodies to 32 kDa bovine inhibin. Miyamoto et al (1986) also reported the presence of a 32 kDa glycoprotein in porcine follicular fluid (pFF), which consisted of 20 kDa and 13 kDa subunits. This confirmed the findings of Rivier et al (1985) and Ling et al (1985). These two subunits have been termed α- and β-subunits. Furthermore, Ling et al (1986a; 1986b) identified two forms of inhibin in pFF: the so called inhibin A and inhibin B. The difference between these two proteins was found to be the amino acid sequences of the smaller subunits which are termed βA and βB.

Leversha et al (1987) isolated a 30 kDa species consisting of 20 kDa and 16 kDa subunits from sheep follicular fluid and the same molecular size of inhibin was also reported in ovine rete testis fluid by Bardin et al (1987). In human follicular fluid (hFF), based on immunoactivity and *in vitro* bioactivity studies, a number of molecular sizes of inhibin, 30 kDa, 36 kDa, 59 kDa and 66 kDa, were identified by Robertson et al, (1990b). The 59-66 kDa was the predominant species as previously reported in bFF (Robertson et al, 1985).

Recently, two immunoactive forms of inhibin with no bioactivity have been identified from bFF. First, a 27 kDa peptide termed pro-αC and secondly, a 25 kDa termed αN-subunit (see Section 1.3). These are presumably the products of proteolysis and rebinding of inhibin α-subunit precursors (Knight et al, 1989; Sugino et al, 1989; Robertson et al, 1989). These immunoactive forms can cross-react with the antibodies against dimeric inhibin as mentioned in Section 1.2.1. At present, the amount of these two biologically inactive forms secreted into the circulation is unknown. Moreover, changes in the immunoactive to bioactive ratio of inhibin over the menstrual cycle have been observed (Robertson et al, 1988a). This has led to a great controversy as to the significance of serum inhibin immunoactivity in different physiological conditions.

The isolation of activins, dimers of the β-subunits of inhibin, is connected to that of inhibin. Ling et al (1985) reported that a fraction obtained during the purification of inhibin from pFF stimulated rather than inhibited FSH secretion from rat pituitary cells. This protein was further purified and proved to be a disulphide-linked homodimer of inhibin βA-subunits (Activin A) or a heterodimer of inhibin βA- and βB-subunits (Activin AB) (Ling et al, 1986b; Vale et al, 1986). The purified protein showed a single band of 24 kDa in molecular size on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions which upon reduction was found to consist of 2 subunits (Ling et al, 1986a; 1986b; Vale et al, 1986). These proteins have opposite actions to that of inhibin. Activin B (βBβB) has never been isolated as a native protein from biological fluids but has been biologically produced using transfection of mammalian tissue culture cells and has similar biological activities and potencies to the other two native activins (Mason et al, 1989). Surprisingly, activin A has been reported to have an identical structure to erythroid differentiating factor (EDF) (Eto et al,1987; Kitaoka et al, 1987).

3. Inhibin structural analysis and homologies

Recently, success in cloning inhibin genes from porcine (Mason et al, 1985; Mayo et al, 1986), bovine (Forage et al, 1986), ovine (Bardin et al, 1987), rat (Esch et al, 1987; Woodruff et al, 1987) and human sources (Mason et al, 1986; Mayo et al, 1986; Stewart et al, 1986) has revealed complete structures of inhibin and activin subunit precursors that were initially deduced from the complementary DNAs (cDNAs). These subunits are separately encoded by different genes. Using Southern blot analysis of somatic cell hybrid DNAs and *in situ* hybridization, it was demonstrated that the human inhibin α and βB genes were assigned to different regions of chromosome 2 whereas the inhibin βA gene was located on chromosome 7 (Barton et al, 1989). The exon-intron structure of the genes coding for individual inhibin subunits are very similar between species. The gene for the human inhibin α-subunit contains an intron of 2.1 kb which splits the coding region while that for the bovine inhibin α-subunit also has a single 1.7 kb intron. An intron of 10 kb has been demonstrated in the βA gene of human and bovine species (Stewart et al, 1986) whereas the full length sequence of the human βB gene contains an intron of 2.5 kb (Mason et al, 1989). This conservation of genomic structure is also supported by a stong structural homology of protein sequences, suggesting that they are derived from one ancestral gene as a result of duplications.

Significant homology exists between the structures of the α- and β-subunits within and between a number of species (Mason et al, 1985; Forage et al, 1986; Woodruff et al, 1987; Leversha et al, 1987). The precursors of porcine, human and bovine inhibin subunits and their cDNAs have been demonstrated (Mason et al, 1985; 1986; Mayo et al, 1986; Forage et al, 1986). The amino acid sequence of the α-subunit precursor shows 85% homology between humans, pigs and cattle (Mason et al, 1986). The βA and βB protein precursors show 70% homology within and between species (Mason et al, 1985; 1986). These subunit precursors are subsequently processed at sites of paired arginine residues to yield the 43 kDa α-subunit and the 15 kDa β-subunit (Figure 1.1) which are the C-terminal region of its precursor protein. The 43 kDa α-subunit is further processed to a 20 kDa peptide which is the α-subunit of the 32 kDa dimeric inhibin. Whether the cleavage of these proteins occurs before or after dimerization is not known. However, the fact that 58 kDa inhibin is converted to 31 kDa inhibin when in contact with serum suggests that α-subunit cleavage can occur after dimerization (McLachlan et al, 1986a). The porcine and human mature α-subunits consist of 134 amino acids from the C- terminal of their precursors (366

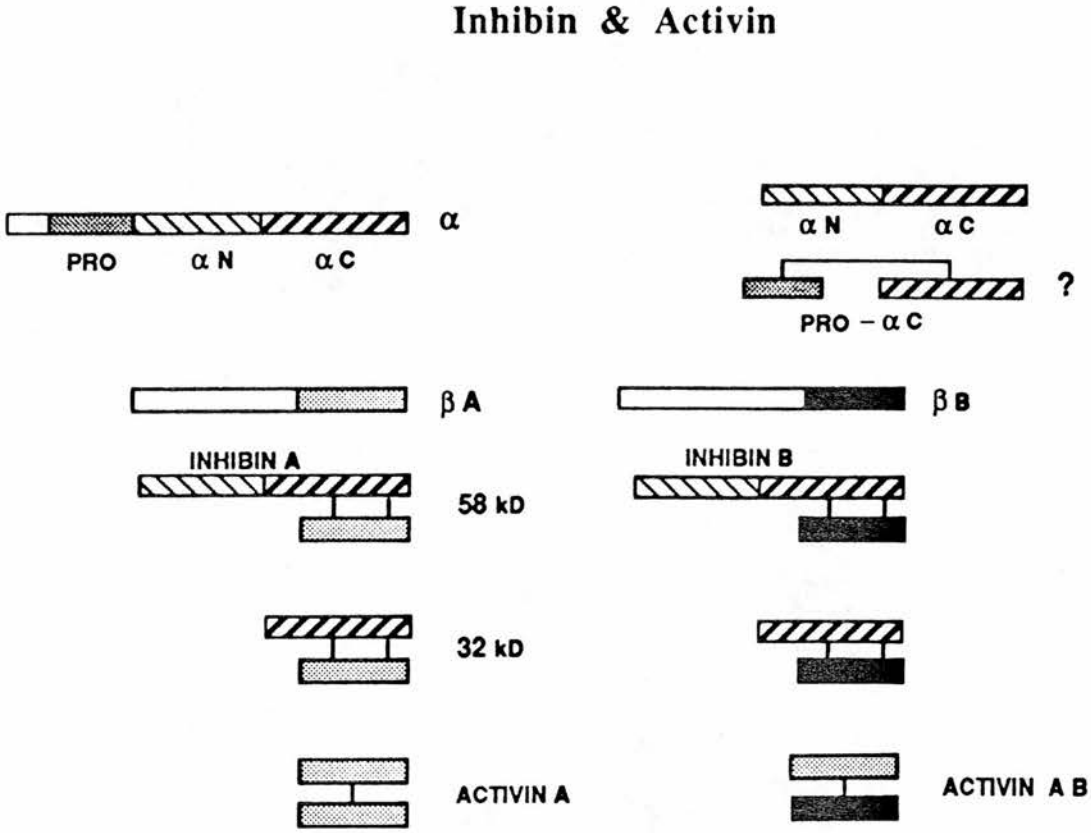

Figure 1.1 Structures of inhibin and activin

amino acids for human, 364 amino acids for porcine). The βA mature subunits in both species are identical, 116 amino acids in length and derived from the C-terminal of their precursors which are 424 amino acids in length. The βB mature subunit is 115 amino acids in length and is derived from the C-terminal of a 407 amino acid protein precursor. In addition, human βA- and βB-subunit show a difference of only one amino acid. There are at least three amino acids which differ between the human, porcine and bovine.

It is reported that inhibin is a glycoprotein due to its binding ability to lentil-lectin and concanavalin A (Jansen et al, 1981; de Jong et al, 1982; Godbout & Labrie, 1984). The amino acid sequence of the human α-subunit precursor predicts three possible glycosylation sites, one at the pro-region and the other two within the mature region, whereas in porcine and bovine only two glycosylation sites are predicted, one located in the pro-region and the other in the mature region. However, only one possible glycosylation site occurs in the pro-region of both β-subunits and no glycosylation site is present in the mature region (Mason et al, 1985; Mayo et al, 1986; Forage et al, 1986). Glycosylation of the mature α-subunit accounts for the difference between molecular weights determined by SDS-PAGE and that based on amino acid weights. The mature inhibin α- and β-subunits which contain seven and nine cysteine residues respectively show a similar distribution of these residues and also some sequence homology around them, further suggesting a common ancestral gene. Interestingly, there is a structural similarity between inhibin β-subunits and other growth factor-related peptides, such as TGF-β (Derynck et al, 1985), MIS (Cate et al, 1986), the decapentaplegic gene complex in *Drosophila* (Padgett et al, 1987) and the VG1 gene in *Xenopus* (Weeks and Melton, 1987). The homology is evidently in the domains surrounding the highly-conserved cysteine residues.

4. FSH-suppressing protein (FSP or follistatin)

FSH-suppressing protein (FSP), a glycosylated single chain polypeptide has been isolated during the purification of inhibin from bFF (Robertson et al, 1987) and pFF (Ueno et al, 1987), and as a product of stellate cells derived from bovine pituitary glands (Gospodarowicz & Lau, 1989). These proteins have inhibin-like bioactivity (5-30% as potent as bovine and porcine 31-32 kDa inhibin) *in vitro*. FSP proteins are different from inhibin, based on their primary and secondary structures and immunoactivity. Bovine FSP consists of three molecular weight proteins of 31 kDa, 35 kDa and 39 kDa whereas 32 kDa and 35 kDa proteins are found in pFF. All forms

have identical amino-terminal amino acid sequences (Robertson et al, 1987; Ueno et al, 1987). The FSP proteins were found to be products of one gene which has arisen through alternative gene splicing (Shimasaki et al, 1988a). This gene has been characterized and sequenced from cDNA libraries of porcine ovaries (Esch et al, 1987), rat ovaries (Shimasaki et al, 1989) and human testes (Shimasaki et al, 1988b). The deduced amino acid sequence of porcine FSP demonstrated homology to a human pancreatic trypsin inhibitor (Esch et al, 1987). Using *in situ* hybridization, the expression of FSP mRNA has been demonstrated in rat ovary, kidney and brain (Shimasaki et al, 1989). The same group have also shown by Northern blot analysis that the expression of mRNA in the ovary can be stimulated by pregnant mare's serum gonadotrophin (PMSG) treatment.

FSP is synthesized locally within the rat follicle to granulosa cells (Findlay et al, 1990). Bovine granulosa cells in culture secrete FSP and its secretion can be stimulated by bovine FSH and cAMP, but not by LH, suggesting that FSP may act as a local regulator of ovarian function (Klein et al, 1991). FSP has effects on differentiation of granulosa cells and delays the process of luteinization (Xiao et al, 1990). However, FSP has also been reported to be an activin-binding protein (Nakamura et al, 1990). Thus, the physiological role of this protein requires further investigation.

5. Sources and physiological significance of inhibin and related peptides in the female

There is strong evidence that inhibin, as measured in peripheral blood during the ovarian cycle, is produced by the ovary. In 1976, inhibin activity was demonstrated in bovine follicular fluid (de Jong & Sharpe, 1976) and later in the follicular fluid of other species (Schwartz & Channing, 1977; Welschen et al, 1977; Channing et al, 1981; Fujii et al, 1983) including human (Chari et al, 1979). Granulosa cells from various species such as rat (Erickson & Hsueh, 1978; Hermans et al, 1982; Croze & Franchimont, 1984; Sander et al, 1984), cow (Henderson & Franchimont, 1981), pig (Channing et al, 1982a) and human (Channing et al, 1984) have been shown to produce inhibin *in vitro*. Furthermore, the production of inhibin from large follicles was greater than from small follicles (Channing et al, 1982b) and also the inhibin content in large follicles was greater than that in small follicles (Tsonis et al, 1983). The localization of inhibin subunits in rat granulosa cells (Cuevas et al, 1987; Merchenthaler et al, 1987; Meunier et al, 1988a) and the expression of inhibin subunit

mRNAs (Meunier et al, 1988a; Rivier et al, 1989; Torney et al, 1989; Woodruff et al, 1988; 1989; Schwall et al, 1990) provide further evidence that follicles are a source of peripheral inhibin during the follicular phase.

In the primate including man the corpus luteum has also been suggested as a major source of ovarian inhibin production. The concentration of immuno- and bioactive inhibin in luteal tissue is very high (Davis et al, 1987) and immunoactive inhibin is produced by human luteal cells in culture (Smith et al, 1992). Using antibodies directed against epitopes on either the α- or β-subunit, inhibin has been localized to the granulosa-lutein cells of the human corpus luteum (Smith et al, 1991). mRNA for the inhibin α-subunit is strongly expressed in human luteal tissue as shown by Northern blot analysis (Davis et al, 1987, Reddi et al, 1990b) and in luteal tissue from the marmoset monkey (Hillier et al, 1989). Recently very convincing evidence for the localization of mRNA for both α-and βA- subunits in the granulosa-lutein cells of the corpus luteum of monkey was obtained using *in situ* hybridisation with cDNA probes (Schwall et al, 1990).

In addition, studies involving luteectomy in the monkey (Basseti et al, 1990) and ovariectomy in women (Illingworth et al, 1991) resulted in a marked fall in circulating inhibin levels; further indicating that the ovary is the main if not the only source of inhibin. More direct evidence for the source of secretion of inhibin by the ovary is available from a study in which its concentration was measured in ovarian venous blood (Illingworth et al, 1991). At all stages of the cycle the concentration of inhibin in ovarian venous plasma is higher than that in peripheral blood, confirming its secretion by the ovary. However, in the follicular phase, the concentration of inhibin in ovarian venous blood draining each ovary is similar. In contrast the concentration of oestradiol in blood draining the ovary containing the dominant follicle is much higher than in that draining the contralateral ovary. These findings indicate that all antral follicles in the ovary make a significant contribution to the secretion of inhibin rather than solely the dominant follicle as is the case with oestradiol. In the luteal phase, the concentration of inhibin is significantly higher in venous blood draining the ovary containing the corpus luteum than in that from the contralateral ovary. Moreover, when the corpus luteum is enucleated or luteotrophic support withdrawn by the administration of an antagonist of GnRH, there is a marked fall in the concentration of inhibin in peripheral plasma (Fraser et al, 1989; McLachlan et al, 1989; Smith & Fraser, 1991). These findings strongly suggest that the corpus luteum secretes inhibin.

5.1 Endocrine role of inhibin

The inhibin hypothesis postulates that inhibin selectively suppresses FSH secretion and this action have been previously shown in many experiments in various species. However, little is known about the role of inhibin in the human female and it is necessary to refer to studies in other species to deduce information from the hormonal profiles of normal or experimental cycles.

In the follicular phase of the menstrual cycle the concentration of FSH falls while that of LH rises slightly (McLachlan et al, 1987a; Reddi et al, 1990a; McLachlan et al, 1990). It might have been expected, therefore, that the secretion of inhibin, which by definition suppresses the secretion of FSH selectively, would increase at this time. Although it was claimed initially (McLachlan et al, 1987a) that there was a progressive rise in inhibin concentration during the follicular phase, further more detailed studies have demonstrated that during the mid-follicular phase of the cycle, when the concentration of FSH is suppressed, there is no change in the concentration of inhibin which remains constant until 2 or 3 days before ovulation. In contrast, the concentration of oestradiol rises progressively throughout the follicular phase from the time that the ovulatory follicles become dominant (about Day 6). In the 48 hours prior to the LH peak the concentration of inhibin rises, falls slightly at around the time of ovulation and then rises again to reach a peak in the mid-luteal phase of the cycle. In the few days prior to the onset of menstruation, the concentration of inhibin falls coincidental with the fall in the concentration of progesterone and oestradiol (Roseff et al, 1989). At the same time the concentration of FSH rises, strongly suggesting that inhibin is secreted by the corpus luteum together with oestradiol and progesterone, and suppresses the secretion of FSH during the luteal phase. The pattern of inhibin secretion is very similar in the non-human primate (Fraser et al, 1989; Basseti et al, 1990; Smith et al, 1990; Webley et al, 1991). Inhibin levels are low during the follicular phase and increase following the LH surge to reach a maximum during the luteal phase.

It is likely that, as in the sheep and the rat, inhibin complements oestradiol in regulating the secretion of FSH (Rivier & Vale, 1989; Baird et al, 1990). As there is little change in the concentration of inhibin in the follicular phase, it is unlikely to be responsible for the decline in the concentration of FSH at this time which is due to the increased secretion of oestradiol by the dominant follicle. Rather, inhibin contributes to the overall level of negative feedback, the amount reflecting the total number of

antral follicles in the ovaries. Thus, the rise in FSH concentration which occurs premenopausally is associated with reduced levels of inhibin (MacNaughton et al, 1990). In the luteal phase the combined secretion of inhibin and oestradiol suppresses the secretion of FSH to a level below the threshold necessary to activate and sustain the growth of large antral follicles. Thus all follicles > 5mm in diameter found during the luteal phase of the menstrual cycle are atretic.

5.2 Paracrine and autocrine role of inhibin and activin

Activin (and TGF-β) enhances the ability of FSH to induce aromatase activity by immature rat granulosa cells, an effect which is partially reversed by adding inhibin (Ying et al, 1986; Ying, 1988). Similar results were observed in rat granulosa cells by Hutchinson et al (1987) who also noted a suppression in progesterone production. The effect of porcine inhibin on reversing the enhancement of FSH-induced aromatase activity by activin, could not be confirmed using bovine inhibin (Hutchinson et al, 1987). Experiments involving the effects of inhibin on granulosa cells are confounded by the production of endogenous inhibin by the granulosa cells. Thus, in contrast to the well characterised effect of activin on granulosa cells, the role of inhibin is uncertain.

However, inhibin appears to have well-defined actions on theca cells. In rat theca cell preparations inhibin enhanced LH-induced androgen production, an effect which was attenuated by activin (Hsueh et al, 1987). Similar findings were observed in human theca cells cultured in serum-free medium (Hillier et al, 1991a; 1991b). Recombinant human inhibin, in concentrations which are within the range found within the follicle, markedly enhanced the action of LH and insulin-like growth factor (IGF) in stimulating the production of androstenedione and dehydroepiandrosterone, but not progesterone, when added to theca cells in culture (Hillier et al, 1991a). In the absence of LH or IGF, inhibin alone had very little action. In contrast, activin suppressed the basal production of androgens in this system and inhibited the stimulatory effect of inhibin (Hillier et al, 1991b). These findings suggest a mechanism by which the granulosa cells could influence thecal cell function. Furthermore, theca cells of the follicle also probably influence the growth and differentiation of granulosa cells. TGF-β, which is structurally related to activin, causes a dose-dependent increase in aromatase activity and inhibin production, an effect which is additive to that of FSH (Zhiwen et al, 1988). Although rat and bovine thecal tissue cultured *in vitro* produce

TGF-β (Skinner et al, 1987) it is not yet known whether activin is produced by theca cells as well as granulosa cells.

5.3 Control of inhibin production

While there is strong evidence to suggest that FSH can stimulate inhibin production by the ovary, the exact mechanism is not entirely understood. Injection of PMSG or FSH into rats, sheep or women results in a large increase in the concentration of immuno- and bioactive inhibin in the ovaries and in serum (Lee et al, 1981; McLachlan et al, 1986a; Buckler et al 1989; Tsonis et al, 1988a). In the study by Tsonis et al (1988a), the concentration of bioactive inhibin was very closely correlated with the rise in the concentration of oestradiol and with the number of large antral follicles. However, in the short term the concentration of FSH appears to have little influence on the secretion of inhibin by the ovary. In the sheep, inhibin secretion is unaffected for up to 12 hours after injecting FSH in amounts which double the basal concentration (Campbell et al, 1991). Inhibin is secreted by the sheep ovary in pulses but each pulse is unrelated to pulses of FSH or LH (McNeilly & Baird, 1989; Campbell et al, 1989). In women there are episodic fluctuations in the concentration of inhibin in peripheral blood during the luteal phase (Nakajima et al, 1990). However, the magnitude of these pulses is modest (<20%) and they are unrelated to episodic pulses of LH or progesterone. Whether these fluctuations in peripheral plasma concentrations of inhibin reflect pulsatile ovarian secretion is difficult to determine due to the long half-life of inhibin.

There is abundant evidence *in vitro* that inhibin production by granulosa cell cultures is dependent on FSH. FSH and testosterone enhance the induction of inhibin production by bovine (Henderson & Franchimont, 1981) and immature human (Hillier et al, 1991c) granulosa cells. The production of inhibin by granulosa cells recovered from large follicles is responsive to LH as well as FSH, while luteinized granulosa cells are only stimulated by LH (Tsonis et al, 1987a). In this latter study it was shown that LH stimulated the production of oestradiol and progesterone as well as inhibin. cAMP and prostaglandin E (which stimulates the production of intracellular cAMP) both mimic the effect of LH and FSH on granulosa cells suggesting strongly that gonadotrophins exert their effect on inhibin production via the adenyl cyclase system linked to specific receptors (Bicsak et al, 1987).

There is evidence both *in vivo* and *in vitro* that a similar mechanism operates within luteal tissue. Injection of hCG *in vivo* prolongs the life span of the corpus luteum and stimulates inhibin secretion (Illingworth et al, 1990). Production of inhibin (and oestradiol and progesterone) by dispersed human luteal cells in culture is stimulated by hCG in a dose-dependent manner (Smith et al, 1992). Whether the same paracrine factors which modulate the responsiveness of the granulosa cells of the follicle to gonadotrophins operate in the corpus luteum is not yet clear.

6. Inhibin and its related peptides in pregnancy

There is an increase in peripheral inhibin concentrations in the luteal phase of the normal menstrual cycle. Many direct and indirect studies have shown that the corpus luteum is a potential source of inhibin (McLachlan et al, 1987c; Tsonis et al, 1988; Illingworth et al, 1991; Smith et al, 1991). The concentration of circulating immunoactive inhibin during pregnancy has been reported in primates. Smith and colleagues (1990) have demonstrated an increase in inhibin levels throughout pregnancy in the common marmoset monkey and this finding has also been observed in the Japanese monkey (Nozaki et al, 1990). In human pregnancy, many studies have revealed an elevation in peripheral bioactive (Qu et al, 1991) and also immunoactive inhibin (McLachlan et al, 1987b; Abe et al,1990; Kettel et al, 1991; Tabei et al, 1991; Smitz et al, 1990; Rombauts et al, 1990). Although the human corpus luteum is likely to be a source of circulating inhibin in the normal cycle (McLachlan et al, 1989; Smith et al, 1991), it is not the only potential source in pregnancy (McLachlan et al, 1987b). Studies have shown inhibin bioactivity in the rabbit (Hochberg et al, 1981) and human placenta (Bandivdekar et al, 1981). The finding in human placenta was confirmed by the presence of bioactive and immunoactive inhibin in term placental extracts (McLachlan et al, 1986b). Inhibin secretion from primary cultures of human trophoblasts was reported by Petraglia et al (1987a) and these authors also demonstrated the presence of inhibin α-subunit immunoactivity in cytotrophoblast cells of the human placenta. This localization was confirmed by Merchenthaler and colleagues in the same year (Merchenthaler et al, 1987).

In human pregnancy, inhibin α-subunit cDNA was isolated from a placental cDNA library (Mayo et al, 1986). Davis and colleagues (1987) have shown the expression of inhibin α- and only βA-subunit mRNAs in term placenta while Reddi and colleagues

(1990b) have demonstrated α- and βB-subunit gene expression in term placenta by Northern blot analysis.

The regulation of inhibin secretion and its effect on the function of other hormones have also been examined. Human chorionic gonadotrophin (hCG) stimulated inhibin secretion from cultured placental cells and this effect could be mimicked by 8-bromo-cyclic AMP, forskolin and cholera toxin (Petraglia et al, 1987a). Inhibin also suppressed the stimulatory effects of GnRH on hCG secretion of trophoblast cells (Petraglia et al, 1987a). However, in tissue culture of placental explants inhibin only suppressed hCG secretion by term placenta, not by first trimester placenta (Mersol-Barg et al, 1990). Furthermore, in human placental cell cultures activin stimulates the production of progesterone, not hCG, but it enhances the stimulation of GnRH on hCG release and the latter effect can be reversed by inhibin (Petraglia et al, 1990).

There is evidence for the presence of inhibin in mid-term amniotic fluid (Sinosich et al, 1991) and activin A in amniotic fluid (Abe et al, 1989) which suggests that the fetus plays a role in the production of inhibin. However, circulating inhibin levels in umbilical cord have been shown to be lower than in maternal blood vessels and there is no difference between umbilical vein and artery levels (McLachlan et al, 1986b; Tabei et al 1991; Kettel et al, 1991).

Apart from human trophoblast, decidual tissue has been reported to be another possible source of inhibin. Petraglia and colleagues (1990) have shown the localization of inhibin-subunits and the expression of the mRNAs for inhibin-subunits in decidual tissue throughout pregnancy.

However, the role of inhibin in pregnancy remains unknown. The significant homology observed between inhibin and the decapentaplegic gene complex in *Drosophila* (Padgett et al, 1987) and the VG1 gene in *Xenopus* (Weeks & Melton, 1987) which are involved in early embryogenesis suggests a possible role for inhibin in embryonic differentiation. Furthermore, inhibin β-subunits have a striking homology with TGF-β which has recently been shown to have an important role in different embryonic events (Heine et al, 1987).

7. Structure of the human placenta

After fertilization, the mature ovum undergoes segmentation or cleavage into blastomeres. During the division of blastomeres the gradual accumulation of fluid results in formation of the blastocyst or fertilized ovum. At this stage the outer cells which are destined to produce the trophoblast can be distinguished from the inner cell mass which are destined to produce the embryo. The blastocyst becomes polarized as it is embedded in the endometrium. The outermost pole, developing towards the endometrium cavity, becomes the chorion laeve, and the innermost pole becomes the chorion frondulosome or the villous and extravillous components of the placenta.

As the blastocyst contacts the endometrium, proliferating trophoblasts invade. Some trophoblasts invade maternal blood vessels, replacing the vascular smooth muscle and endothelium cells resulting in the formation of trophoblast-lined vascular channels. Other trophoblasts are orderly formed into discrete chorionic villous structures. These villous structures (Figure 1.2) consist of an outer layer of syncytiotrophoblasts which are derived from the fusion of mononuclear cytotrophoblasts, cells in the inner layer lying beneath the syncytiotrophoblast layer. These two subdivisions of trophoblasts are distinguishable; the cytotrophoblast consists of individual cells with relatively pale-staining cytoplasm, and in the syncytiotrophoblast, dark-staining nuclei are distributed irregularly within a common basophilic cytoplasm. The cytotrophoblasts are joined to each other and the syncytiotrophoblasts by desmosomes. The trophoblasts lie on a basement membrane surrounding a central core of mesenchymal cells, macrophages and capillary endothelium.

The chorionic villi begin as small buds and grow from a stem into a complex branching structure. As the placenta matures, the short, thick, early stem villi branch repeatedly, forming progressively finer subdivisions and greater numbers of increasingly smaller villi. Each of the main stem villi and its branches constitute a placental cotyledon. The villi in a cotyledon are classified according to their positions in the tree, the type of fetal blood vessels and the connective tissue structure. The stem villous has centrally-located fetal arteries and veins and a rich connective tissue stroma. Intermediate villi branch from the stem villi containing arterioles and venules, and branch into terminal villi. These terminal or tertiary villi have fetal capillaries and a minimal fetal diffusion distance (see Boyd & Hamilton, 1970).

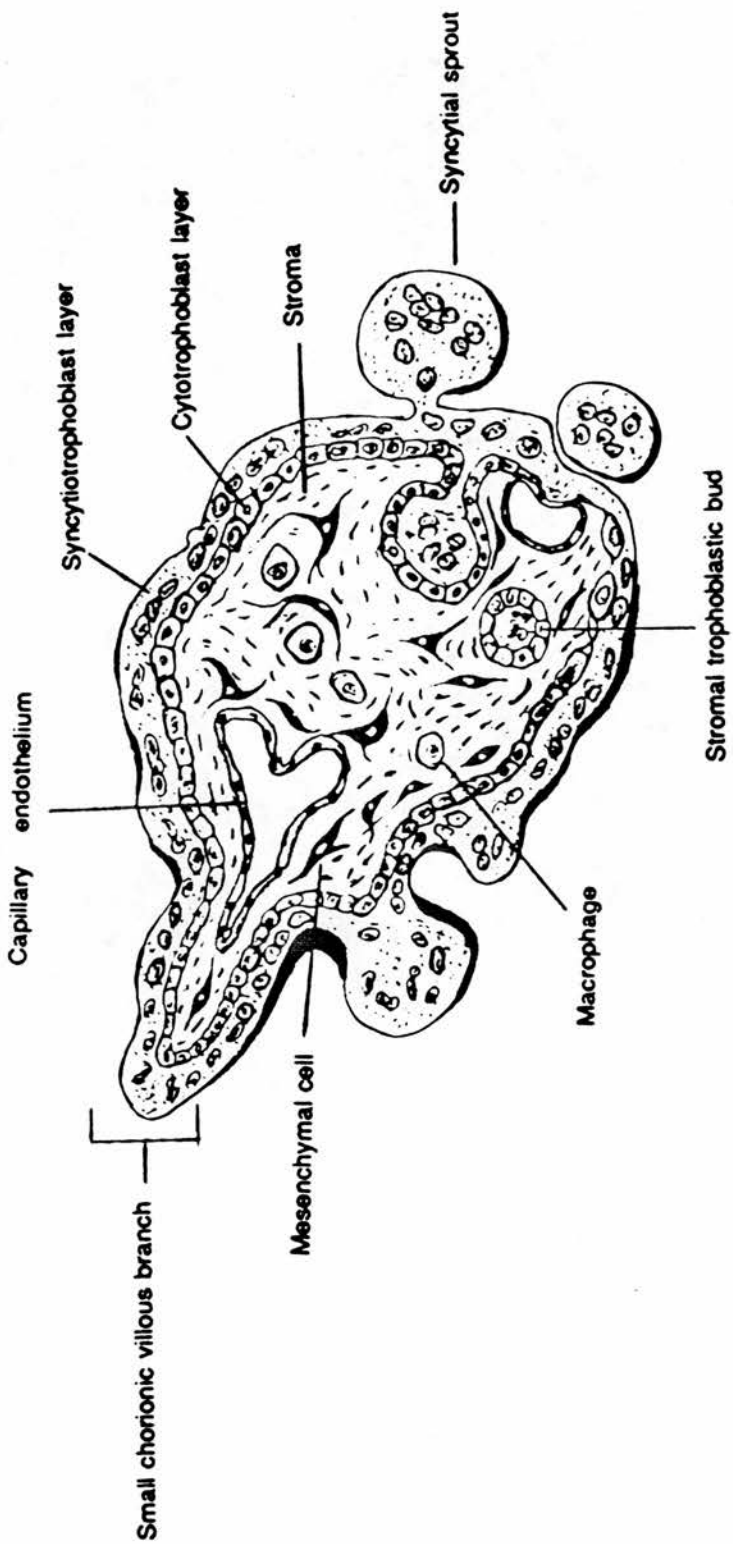

Figure 1.2 Schematic diagram to show structure of the chorionic villus. Modified from Boyd & Hamilton (1970).

During the first trimester, the growth of the placenta is rapidly driven by the multiplication of the cytotrophoblasts. These proliferation cells extend through the tips of the villi, which anchor the placenta to the uterus, as cytotrophoblast columns which then spread out to form the cytotrophoblast shell. This shell is interposed between the villi and uterine decidua. As cytotrophoblast cells mature, they lose mitotic activity and then fuse to form syncytiotrophoblast.

Placental growth slows down during the second and third trimesters, and the relative ratio of syncytiotrophoblast to cytotrophoblast increases dramatically. It has been suggested that the differentiation of cytotrophoblasts to syncytiotrophoblasts encompasses an intermediate state (Kurman et al, 1984a; 1984b). The trophoblasts in this state (so called 'intermediate trophoblasts') are the cells characterized as round, or polyhedral mononuclear cells with larger and hyperchromatic nuclei compared to those of normal villous cytotrophoblasts. The intermediate trophoblast has abundant cytoplasm which is less eosinophilic than that of syncytiotrophoblast. Mononuclear trophoblasts with these characteristics are found in villi and extravillous sites.

The villous trophoblasts are the potential sites of hormone production, but the hormonal expression of these morphologically distinct trophoblasts are different. The cytotophoblast synthesizes the hypothalamic releasing factors secreted by the placenta such as GnRH, CRF and somatostatin (Khodr & Siler-Khodr, 1980; Petraglia et al, 1987b, c; 1989; Robinson et al, 1988; Watkins & Yen, 1980). The syncytiotrophoblast synthesizes chorionic gonadotrophin (CG), chorionic somatomammotrophin (CS) and different forms of growth hormone (Hoshima et al, 1982; Beck et al, 1986; Liebhaber et al, 1989). It is also the most active steroidogenic placental cell type; the aromatiziation of C19 steroids from fetal and maternal origin into C18 phenolic oestrogens takes place in syncytiotrophoblast cells (Fournet-Dulguerov et al, 1987). Both syncytiotrophoblasts and cytotrophoblasts are the site of inhibin-like peptide production (Petraglia et al, 1987a; 1990; Merchenthaler et al, 1987; Tovanabutra et al, 1991).

Extravillous cytotrophoblasts and syncytiotrophoblasts (giant wandering cells) and cytotrophoblasts of the chorion laeve also produces hormones, but the profile of synthesized hormones of these extravillous trophoblasts are different from the villous trophoblasts. The extravillous trophoblastic cells do not react with antibodies to CS, and CG in the same manner as those of villous trophoblasts (Sasagawa et al, 1987). The unique phenotype of extravillous trophoblast revealed by immunocytochemical

studies most likely reflects the impact of their milieu rather than intrinsic differences in the differentiation of villous and extravillous cells. Although it is likely that paracrine/autocrine control is involved in these processes, the factors responsible for the morphological and functional differentiation of the trophoblasts are still unknown.

The placenta is an organ which has dynamic changes in growth rate, structure and cellular composition. Moreover, the function of each cell type may vary during pregnancy (e.g CG is produced by cytotrophoblasts of the blastocyst during implantation). At the beginning of first trimester, the villous cytotrophoblasts do not produce CG but syncytiotrophoblasts secrete vast amounts and the production falls substantially when the pregnancy progresses to term. Studies on the placenta must be viewed carefully and the results must be interpreted with caution.

8. Aims of thesis

In this thesis I shall describe studies aimed at identifying the nature of inhibin and related peptides in human pregnancy.

In Chapter 2, in order to obtain a suitable antibody for monitoring inhibin immunoactivity produced by the placenta in different studies (e.g. the molecular species of inhibin in placental extracts and immunohistochemistry), RIA and Western immunoblot analysis were exploited to test the specific cross-reaction of the available inhibin antibodies to inhibin.

Aiming to investigate inhibin interaction with other hormones and the potential source of peripheral inhibin during pregnancy, the studies in Chapter 3 were designed to measure the concentration of inhibin, hCG, oestradiol and progesterone in plasma of pregnant women from conception through implantation until delivery.

In Chapter 4 the experiments were designed, first to exploit the available *in vitro* sheep pituitary cell bioassay for measuring and characterizing bioactive inhibin in placental extracts from different stages of pregnancy, secondly to observe inhibin immunoactivity in placental extracts and changes in the immunoactivity to bioactivity ratio during pregnancy and lastly to observe the relationship between placental immunoactive inhibin and hCG content in placental extracts during pregnancy.

To obtain information on inhibin in amniotic fluid during pregnancy the studies in Chapter 5 were performed, first to use three available antibodies which recognise different parts of the inhibin molecule, secondly to examine the *in vitro* bioactivity of amniotic fluid inhibin and lastly to verify the dimeric inhibin bioactivity by immunoneutralization of the bioactivity using an antibody raised against the human synthetic inhibin α-subunit.

The cellular localization of inhibin α- and β-subunits in human placenta was investigated throughout pregnancy by the studies in Chapter 6 using immunohistochemical techniques.

The studies in Chapter 7 were aimed to investigate inhibin gene subunit expression in the human placenta and also to examine the different expression of these subunit mRNAs at various stages of pregnancy. Finally, to further our understanding on the site of inhibin subunit protein production and gene expression, the study in Chapter 8 was carried out to investigate the cellular localization of inhibin gene expression using the *in situ* hybridization technique.

CHAPTER 2

Characterization of antibodies used for radioimmunoassay and immunocytochemistry by Western blot analysis of inhibin and related peptides

2.1 Introduction

At present there are two types of antibodies to inhibin available. The first type is an antibody against purified bovine 58 or 32 kDa inhibin (McLachlan et al, 1986a; 1987a; Robertson et al, 1988a; 1988b) and partially-purified porcine or bovine inhibin (Hasegawa et al, 1987; 1988). The radioimmunoassays (RIAs) developed using these antibodies are very sensitive, thus allowing the measurement of low levels of inhibin in the circulation of human and other species.

The second type of antibody is raised to a range of synthetic peptides representing the amino terminal region of the mature α-subunit of porcine 1-25 or 1-26 inhibin (Rivier et al, 1986; McNeilly et al, 1989; Schanbacher et al, 1988; Sharpe et al, 1988) and human 1-23, 6-30 or 1-32 inhibin (McNeilly et al, unpublished data; Groome et al, 1990; Saito et al, 1989; Sinosich et al, 1991; Mersol-Barg et al, 1990). The RIA with this α-subunit antibody has been widely used to measure inhibin in different preparations such as gonadal extracts, culture media of gonadal cells or tissues and also in serum of some species.

RIAs using either of these antibodies do not cross-react with other proteins in the inhibin family (activin, TGF-β and MIS) but have strong cross-reactivity with another immunoactive inhibin species, pro-αC, which has been found in bovine and porcine follicular fluids (Robertson et al, 1989; Sugino et al, 1989; Schneyer et al, 1990).

Apart from their advantages for RIA, these antibodies are also useful in identifying and characterizing inhibin in preparations separated by electrophoretic procedures in particular Western immunoblot analysis. This technique has been exploited to demonstrate the molecular species of inhibin in bovine follicular fluid (Miyamoto et al, 1986; Knight et al, 1989), human serum (Schneyer et al, 1990) and culture media from gonadal cells (Grootenhuis et al, 1990).

The experiments in this chapter were designed to use RIA and Western blot analysis to test the specificity of the available inhibin antibodies with inhibin immunoactivity in placental extracts from various stages of pregnancy and to compare this with ovarian follicular fluid inhibin. It had been hoped to use these antibodies to characterize inhibin-like peptides in placental extracts. In the event it was not possible to obtain sufficient quantities of purified protein. However, the antibody and methods obtained were useful for immunohistochemistry.

2.2 Characterization of antibodies by radioimmunoassay

2.2.1 Materials & Methods

Placental extracts

Placental tissue was obtained from pregnancy terminations at 7 weeks and 16 weeks, and from full term pregnancy. The placental cotyledons were cut off and washed extensively with phosphate-buffered saline (PBS; see Appendix 1) to remove blood. The tissues were homogenized in two volumes of PBS with a Polytron homogenizer. The homogenate was then centrifuged at 1,000 g for 15 min at 4°C and the supernatant collected and recentrifuged at 100,000 g for 1h at 4°C. The supernatant was stored at -70° C until the assays were performed.

Human follicular fluid (hFF)

hFF was obtained from patients undergoing ovarian stimulation for *in vitro* fertilization during oocyte retrieval.

Antibodies

Antibodies to different fragments of inhibin α- and βA-subunits were purchased or kindly provided from different sources (as summarized in Table 2.1 with accompanying details). The assays using these antibodies were developed with advice from Dr. Alan McNeilly and help from Ms. Wendy Crow.

Table 2.1 Summary of the antibodies with the sources and immunogens

Name	Source	Immunogen
Y29	Sheep	Human α-(1-23)-peptide
6DF5	Sheep	Human α-(1-23)-peptide
S55	Sheep	Porcine α-(1-26)- peptide
R187	Rabbit	Human βA-(97-112)-peptide
Monash antibody	Rabbit	31kDa bovine inhibin

1) Y29 is the antibody raised in sheep against human inhibin α-(1-23)-NH_2 (Peninsula Laboratories, San Carlos, CA) conjugated with rabbit gammaglobulin by the carbodi-imide method and produced by Dr. Alan McNeilly (McNeilly et al, 1989).
2) 6DF5 is the antibody raised in sheep against human inhibin α-(1-23)-NH_2 fragment (kindly prepared by Dr. Rodney Kelly) conjugated with rabbit gammaglobulin by Dr. Alan McNeilly.
3) S55 is the antibody raised in sheep against porcine inhibin α-(1-26)-NH_2 (kindly provided by Dr. Jean Rivier) conjugated with ovalbumin .
4) R187 is the antibody raised in rabbit against human inhibin β-(97-112)-NH_2 conjugated with Ultrasyl-D resin by Dr. Rodney Kelly.
5) Monash antibody (no. 1989) is the antibody raised in rabbit against 31kDa bovine inhibin using the method described by McLachlan et al (1986a).

Radioimmunoassays

1) Inhibin assay using the Y29 antibody
Reagents:
Assay buffer: 0.075 M phosphate buffer, pH 7.3, containing 0.15 M NaCl, 1% bovine serum albumin (RIA grade, Sigma) and 0.01% thiomersal (BDH).
Standards: Human inhibin α-subunit (1-23)-NH_2 (Peninsula Laboratories) was diluted in assay buffer to obtain the working range from 3.9 to 2000 pg/100μl.
Antiserum: Y29 was diluted with assay buffer at the dilution of 1 in 60,000.
Tracer: The ^{125}I-human inhibin α-(1-23)-NH_2 was prepared by a modification of the chloramine-T method (McNeilly et al, 1989). The iodination was performed as follows:
A peptide aliquot (1μg/50μl in 0.1M phosphate buffer, pH 7.5) and 5μl of Na^{125}I (10 mCi/100μl, Amersham) were pipetted into a 1.5ml Eppendorf tube. 10 μl of

chloramine-T (1mg/ml in 0.5 M phosphate buffer, pH 7.5) was added and the reaction solution mixed and left to stand for 60 seconds. The reaction was stopped by the addition of 10 µl of sodium metabisulphite (1mg/ml in 0.05M phosphate buffer, pH 7.5) and 430 µl 0.05M phosphate buffer containing 0.05% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS, Sigma). The mixture was chromatographed to separate ^{125}I-peptides from free ^{125}I using a 30x1 cm Sephadex G-25 column which has been previously equilibrated with 0.05M phosphate buffer containing 0.05% CHAPS (eluent buffer). 0.8 ml fractions were collected and the elution profile is shown in Figure 2.1. The two fractions at the top of the peptide peaks were kept as the stock tracer.

Assay procedure:

The assay was performed by incubating 100 µl sample, 100 µl of the diluted antibody (1:60,000) in assay buffer and 100 µl of ^{125}I- labelled antigen (15,000 cpm/100µl in assay buffer) with 200 µl assay buffer at 4°C for 24 h. The antigen-antibody complexes were precipitated by adding 100 µl of diluted normal sheep serum (1:500) and 100 µl of diluted donkey anti-sheep/goat globulin (1:10) which were both kindly donated by the Scottish Antibody Production Unit (SAPU), Lanarkshire, Scotland. After 18 h further incubation at 4°C, 1 ml of 0.9% NaCl was added and the assay tubes centrifuged at 1,600g for 20 min at 4°C. The supernatant was discarded and the precipitate counted in a gamma counter (Pharmacia-Wallac 1261 multigamma manual gamma counter). The data were analysed using the 'Assay Zap Universal Assay Calculator' written by Dr. P.L. Taylor for Elsevier, Biosoft, U.K. The sensitivity of the assay was 15 pg/100µl and the coefficients of variation of intra-assay and inter-assay were 3.9% and 11.2% respectively.

2) Inhibin assay using the 6DF5 antibody

Assay buffer, *standards*, *tracer* and *assay protocol* were as described in the assay with the Y29 antibody.

Antiserum: 6DF5 was diluted with assay buffer to 1 in 10,000.

3) Inhibin assay using the S55 antibody

Assay buffer, *standards* and *assay procedure* were as described in the assay with the Y29 antibody.

Antiserum: S55 antibody was diluted with assay buffer at the dilution of 1 in 40,000.

Tracer: The ^{125}I-porcine inhibin α-(1-23)-NH_2 was prepared by the modified chloramine-T method (McNeilly et al, 1989). The iodination was performed as described above. The iodination profile is shown in Figure 2.2.

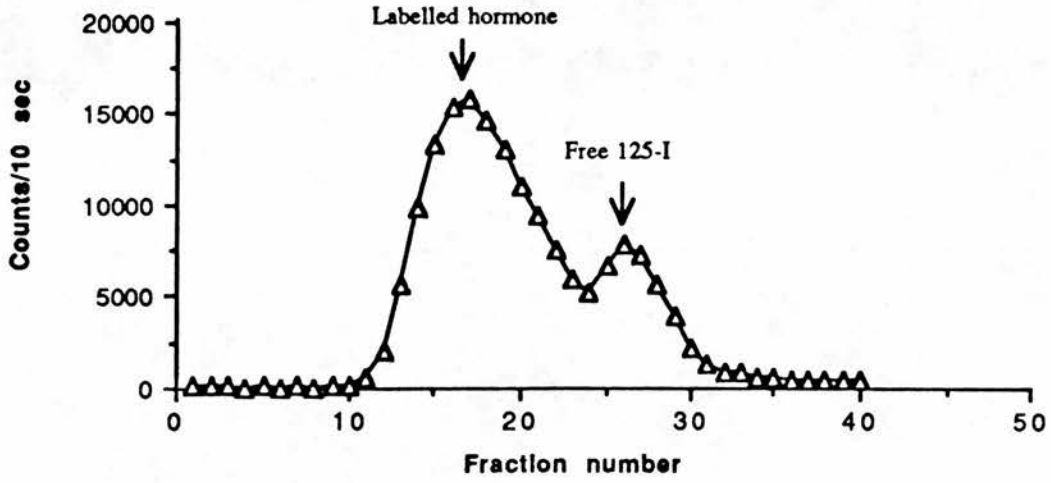

Figure 2.1 Representative elution profile of human inhibin α-(1-23)-NH_2 iodination.

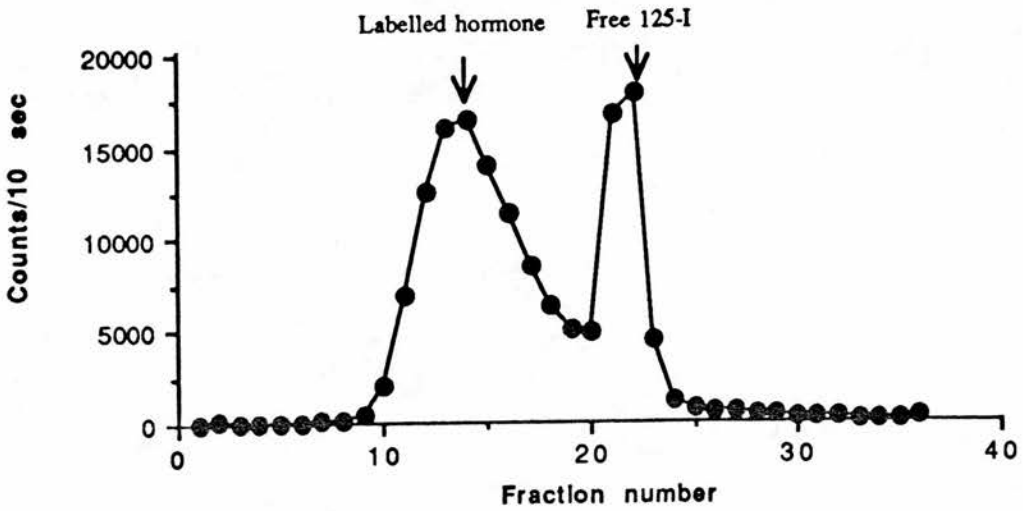

Figure 2.2 Representative elution profile of porcine inhibin α-(1-26)-NH_2 iodination.

The assay protocol is the same as for the inhibin assay with the Y29 antibody.

4) Inhibin assay using the R187 antibody

Reagents:

Assay buffer: 0.075 M phosphate buffer, pH 7.3, containing 0.15 M NaCl, 1% bovine serum albumin (RIA grade, Sigma) and 0.01% thiomersal (BDH).

Standards: Human inhibin βA-subunit (97-112)-NH_2 was diluted in assay buffer to obtain a working range from 20 to 5,000 pg/100µl.

Antiserum: R187 antibody was diluted with assay buffer to 1 in 1,000.

Tracer: The ^{125}I-human inhibin βA-(97-112)-NH_2 was prepared as described for human (1-23) α-inhibin. The iodination profile is shown in Figure 2.3.

Assay procedure:

The RIA was performed by incubating 100 µl of sample or standard with 100 µl of diluted first antibody (1:1000) and the incubation volume adjusted to 400 µl with 200 µl assay buffer. The assay tubes were incubated at 4°C for 24 h, and 100 µl of labelled antigen (15,000 cpm) was added. The incubation proceeded for a further 24 h at 4°C. 100 µl of diluted normal rabbit serum (1:500) and 100 µl of diluted donkey anti-rabbit immunoglobulins (1:40) provided by SAPU were added to separate the antigen-antibody complexes. After incubation for a further 18 h at 4°C, the assay tubes were centrifuged and the precipitates counted. The data were calculated using the same computer programme described above.

5) Inhibin assay with the Monash antibody

Reagents:

Assay buffer: 10mM phosphate buffer, pH 7.3, containing 0.15 M NaCl, 0.5% bovine serum albumin (RIA grade, Sigma) and 0.1% sodium azide (BDH).

Human recombinant inhibin A: was purchased from NIH (NIH rhINH-R-90/1).

Standards: A partially-purified human follicular fluid was prepared using the method described elsewhere (Reddi et al, 1990a). The activity of this inhibin-containing preparation was 23.0 ± 1.2 U/l (mean ± SEM) which was assayed against ovine rete testis fluid standard using the *in vitro* sheep pituitary cell bioassay (Tsonis et al, 1988). The range of standards was from 3.6 to 230 mU/100µl. An arbitrary unit of this standard was different from the partially purified human follicular fluid used by the Monash group (Robertson et al, 1988b) because they were standardized against the same ovine rete testis fluid standard in different bioassay systems. The rat pituitary cell bioassay was not as sensitve as the sheep pituitary cell bioassay as mentioned elsewhere (Tsonis et al, 1988). Thus, the different response of sheep and rat pituitary

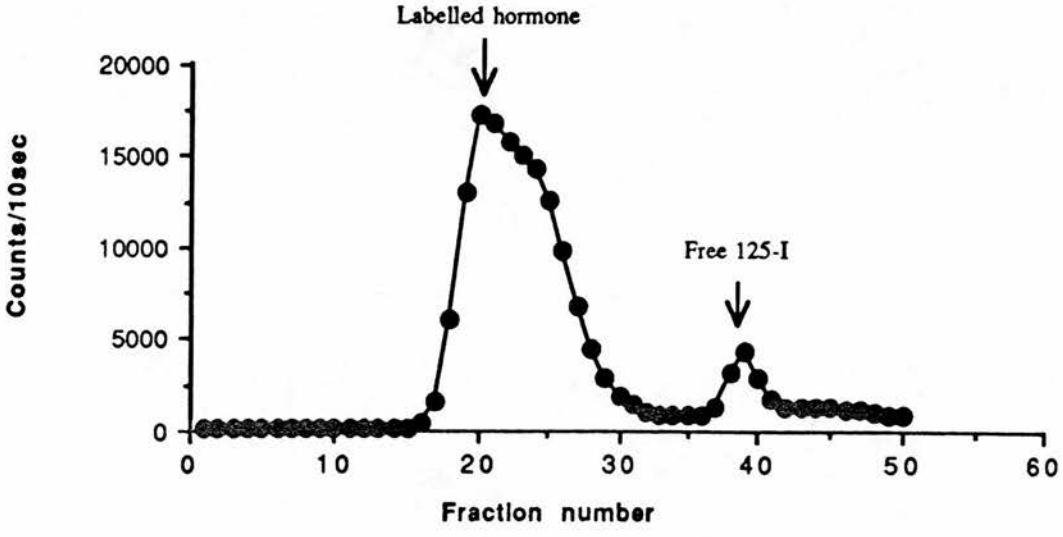

Figure 2.3 Representative elution profile of human inhibin βA-(97-112)-NH_2 iodination.

cells to human inhibin made an arbitrary unit of these two standards incomparable. However this in-house standard was standardized against human recombinant inhibin A (NIH rhINH-R-90/1) in radioimmunoassay and one milliunit of this standard was equal to 3.5 pg recombinant inhibin A (Smith et al, unpublished data).

Antiserum (no. 1989): The antiserum was purchased from Monash University, Melbourne, Australia and diluted to 1 in 3,000 with assay buffer containing normal rabbit serum (SAPU) at a dilution of 1 in 600.

Tracer: Bovine 31 kDa inhibin (purchased from Monash University) was iodinated using the chloramine-T method and further purified by the method described by McLachlan et al (1986a) with slight modifications. The iodination procedure was carried out as follows:

20 µl (1-2 µg) of 31 kDa bovine inhibin, 20 µl of 0.5 M phosphate buffer (pH 7.5) and 10 µl of $Na^{125}I$ (10 mCi/100 µl, Amersham) were pipetted into a 1.5ml Eppendorf tube. 80µl of chloramine-T in 0.5 M phosphate buffer (400 µg/ml) was added and the reaction was left to proceed for 60 seconds. 40 µl of sodium metabisulphite (3mg/ml in distilled water) and 800 µl of 0.02M phosphate buffer containing 0.1% BSA were added to terminate the reaction. The mixture was then subsequently purified by applying it to a PD10 column (Sephadex G-25, Pharmacia) which was pre-equilibrated with 30ml of 0.02M phosphate buffer, pH 6.0, containing 0.1% BSA (eluent buffer A). Fractions of 0.5 ml were collected and the elution profile is shown in Figure 2.4.

The protein peak fractions were pooled and made up to 20 ml with eluent buffer A, then applied to a column containing 400 µl pre-equilibrated Red Sepharose (Pharmacia) which was prewashed with 10 ml eluent buffer A. The effluent was collected and reapplied to the column. After the effluent had run through, the column was washed with 3x1ml of eluent buffer A. The iodinated inhibin was eluted with 2x1ml of eluent buffer A containing 1M KCl (BDH) and 4 M Urea (BDH). In order to remove the KCl/Urea, the collected fractions were applied to PD10 columns which were previously equilibrated with 30 ml of 10 mM phosphate buffer, pH 7.3, containing 0.15 M NaCl, 1% BSA and 0.1% Triton X-100 (eluent buffer B). Following the sample, 1.5 ml of eluent buffer B was run through the column and the effluent was discarded. A further 3 ml of the eluent buffer B was applied and the eluate containing iodinated bovine inhibin from each PD10 column was collected and pooled as the stock tracer.

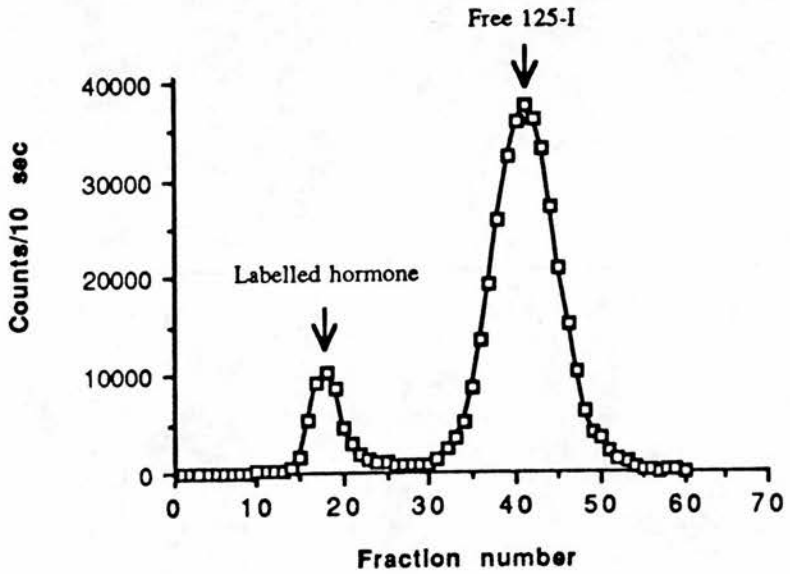

Figure 2.4 Representative elution profile of 31kD bovine inhibin iodination before further purification.

Assay procedure:

The assay was set up by incubating 100 µl of sample or standard, 100 µl of diluted antibody and 200 µl of assay buffer for 24 h at room temperature. 100 µl of ^{125}I-bovine inhibin (15,000 cpm/100µl in assay buffer containing 0.1% Triton X-100) was added and the reaction mixture was incubated for a further 24 h at room temperature. After adding 100 µl of diluted donkey anti-rabbit immunoglobulin (1:64 in assay buffer) provided by SAPU, the incubation was continued for a further 16-18 h at 4°C. 1.5 ml of 0.9% NaCl containing 4% polyethyleneglycol 6000 (BDH) and 0.1% Triton X-100 was added and the assay tubes centrifuged at 2,500g for 45 min at 4°C. The supernatant was aspirated and the precipitate counted. The sensitivity of the assay (ED_{20}, ED_{50} and ED_{80}) was 7.6, 22.1 and 50.4 mU/100µl (n=20) respectively and the coefficients of variation of intra-assay and inter-assay near ED_{50} were 6.6 and 11.5% respectively.

2.2.2 Results

Y29 antibody

This antibody demonstrated the inhibin immunoactivity of placental extracts. Parallel dilution curves of human inhibin α-(1-23)-NH_2 standard and placental extracts from 7 weeks, 16 weeks and delivery were obtained (Figure 2.5a). The same parallelism was also obtained from human follicular fluid (Figure 2.5b).

6DF5 antibody

The RIA using 6DF5 antibody which was raised against human inhibin α-(1-23)-NH_2 demonstrated parallelism in the dose-response lines between placental extracts from different stages of pregnancy, but not the standard (Figure 2.6).

S55 antibody

The dose-response lines of human inhibin α-(1-23)-NH2 and hFF were parallel in the RIA with the S55 antibody, raised against porcine α-(1-26)-NH_2), (Figure 2.7a) but this parallelism was not observed with placental extracts. However, the dilution curves of placental extracts from 7 weeks and 16 weeks of pregnancy, and delivery were parallel (Figure 2.7b)

R187 antibody

This antibody, which is raised against the inhibin βA-subunit fragment, demonstrated specific cross-reactivity to recombinant activin A at high concentrations from 100 ng upwards (Figure 2.8a). Human follicular fluid and placental extracts from various stages of pregnancy showed low immunogenic activity (Figure 2.8b).

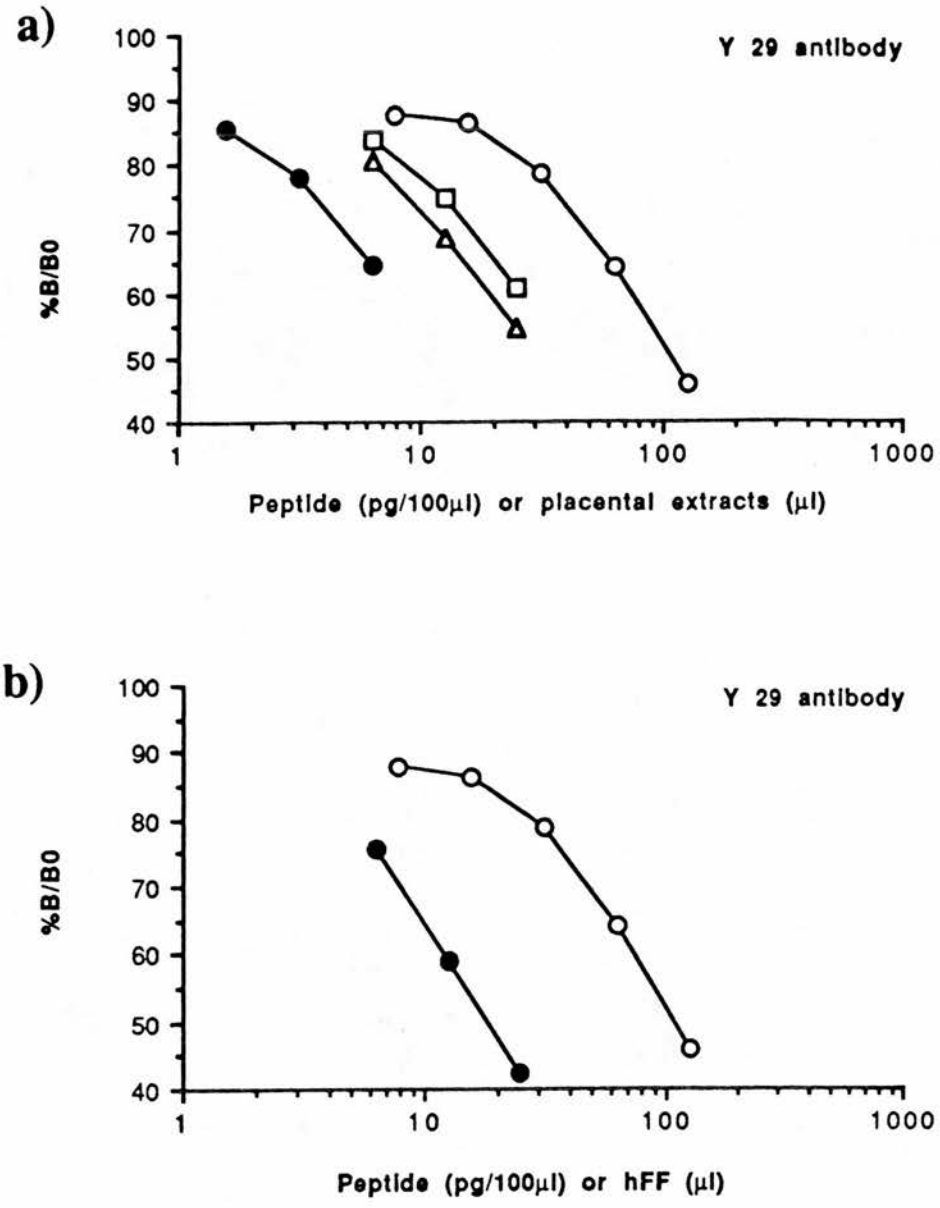

Figure 2.5 (a) Dose-response lines of synthetic human inhibin α-(1-26)-NH_2 (O) and placental extracts from 7 weeks (●), 16 weeks (Δ) and delivery (□) demonstrating parallelism in RIA using Y29 antibody. (b) Parallelism between the dilution curves of human inhibin α-(1-26)-NH_2 (O) and human follicular fluid (●).

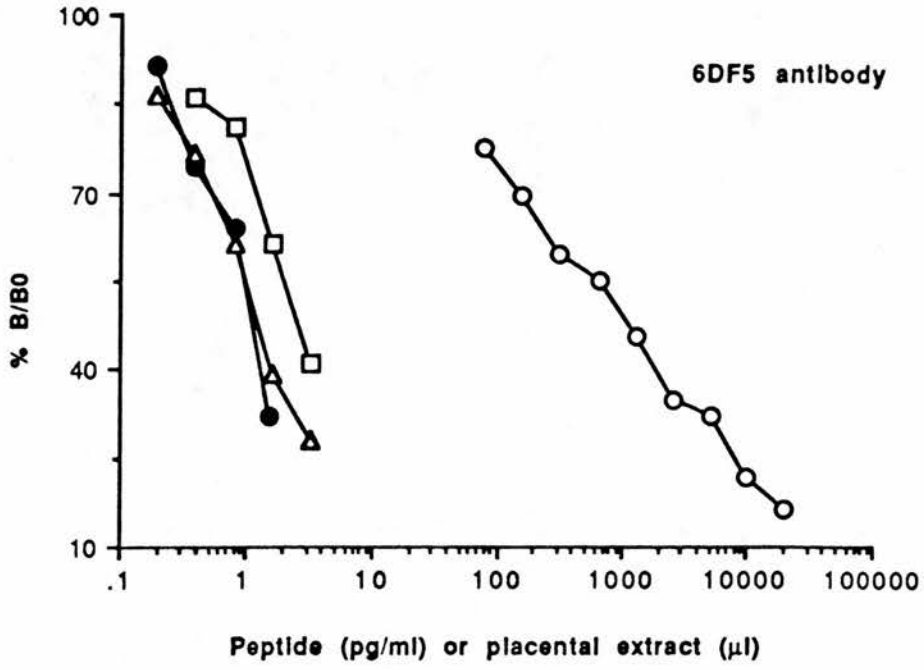

Figure 2.6 Dose-response curves of human inhibin α-(1-26)-NH_2 (O) and placental extracts from 7 weeks (●), 16 weeks (Δ) and delivery (□).

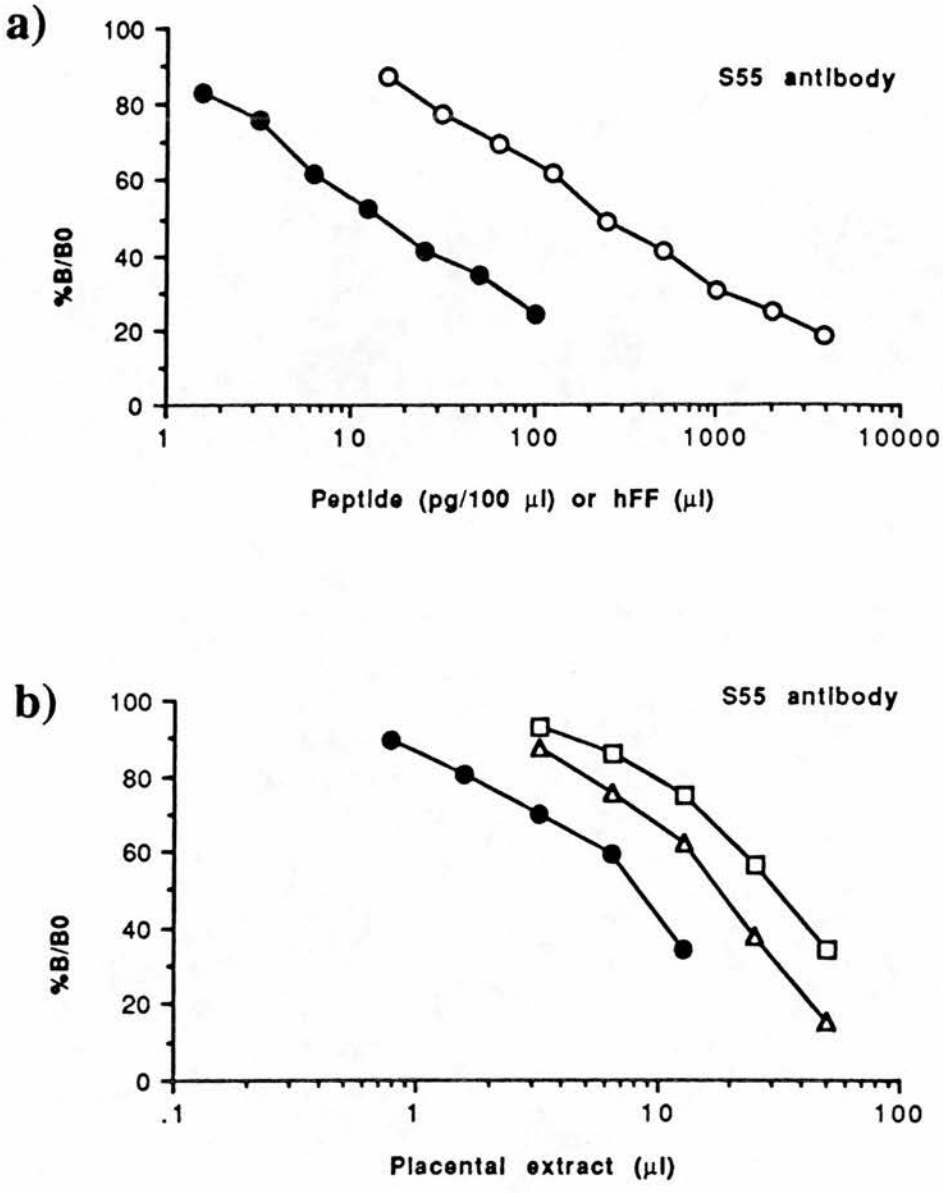

Figure 2.7 (a) Dose-response curves of synthetic human inhibin α-(1-26)-NH_2 (O) and human follicular fluid (●). (b) Dose-response curves of placental extracts from 7 weeks (●), 16 weeks (Δ) and delivery (□).

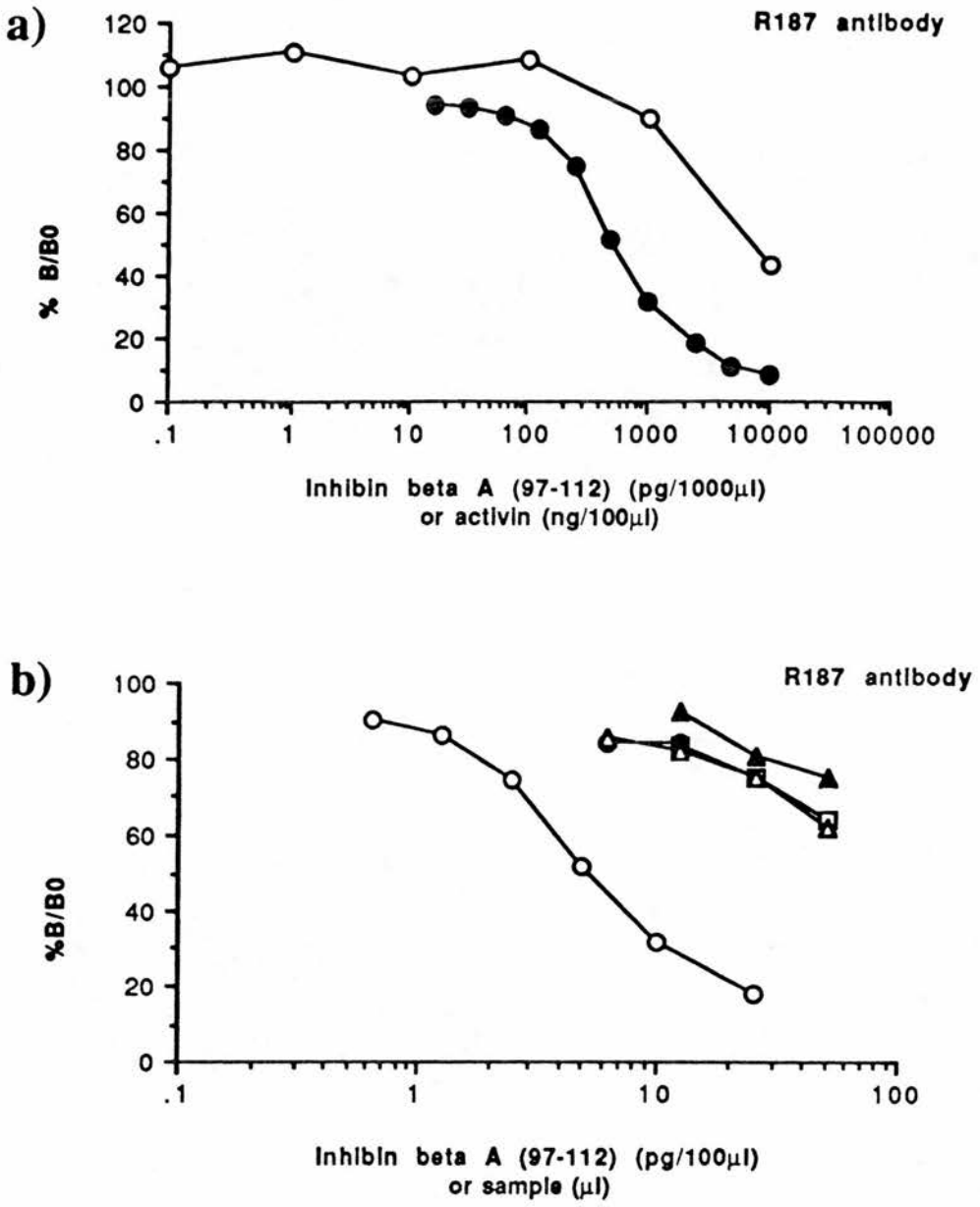

Figure 2.8 (a) Dose-response curves of human inhibin βA-(97-112)-NH_2 (●) and recombinant activin A (O) demonstrating parallelism. (b) Dose-response curves of human inhibin βA-(97-112)-NH_2 (O), h-FF (▲) and placental extracts from 7 weeks (●), 16 weeks (Δ) and delivery (□) demonstrating low immunoactivity in RIA with R 187 antibody. Placental extracts from different stages of gestation showed similar potency in displacing the tracer and the curves are superimposed one and another.

Monash antibody (no. 1989)

The RIA with this antibody showed about 10-fold greater sensitivity to placental extracts than the Y29 antibody. The partially-purified hFF standard curves and the dilution curves of placental extracts are parallel and this parallelism was also observed with human recombinant inhibin A (Figures 2.9a and 2.9b)

2.2.3 Discussion

The results in this section show that the antibody against 31kD inhibin is the most sensitive in measuring inhibin in placental extracts. This antibody has been reported to specifically cross-react with inhibin in numerous biological fluids including term placental extracts (see review by Robertson, 1990). Little is known of placental function during pregnancy and the species of inhibin secreted by the placenta is unidentified. Thus, the results demonstrating parallelism of placental extract dilution curves from different stages of pregnancy and partially-purified hFF standard show the specificity of the Monash antibody and the similarity of inhibin species produced during gestation.

The present data also show parallelism between the displacement of placental extracts from different stages of gestation and hFF in RIAs using the Y29 antibody. The displacement curves are similar to those obtained using the Monash antibody. This suggests specificity of both antibodies which is not seen in RIAs with the 6DF5, S55 and R187 antibodies. The Monash antibody, which is raised against intact bovine dimeric inhibin, has been shown to cross-react with free α-subunit or pro-αC (Robertson et al, 1989; Sugino et al, 1989) suggesting that this antibody recognizes the α-subunit. Y29 antibody is directed against the inhibin α-subunit. Thus, these antibodies detect intact inhibin and also the α-subunit or pro-αC. Nevertheless, recombinant inhibin A shows parallel displacement with hFF standards and placental extracts in the RIA using the Monash antibody. This suggests that the same species of immunoactive inhibin as recombinant inhibin A is present in hFF and placental extracts.

The antibody R187, raised against human inhibin βA(97-112)-NH_2, also has immunogenic activity. The sensitivity of the assay with the inhibin βA-peptide fragment standard is 170 pg/tube and with recombinant activin A is 100 ng/tube. Recombinant activin A dilutes in parallel with the inhibin βA-peptide fragment whereas the same parallelism is not shown with placental extracts and human follicular

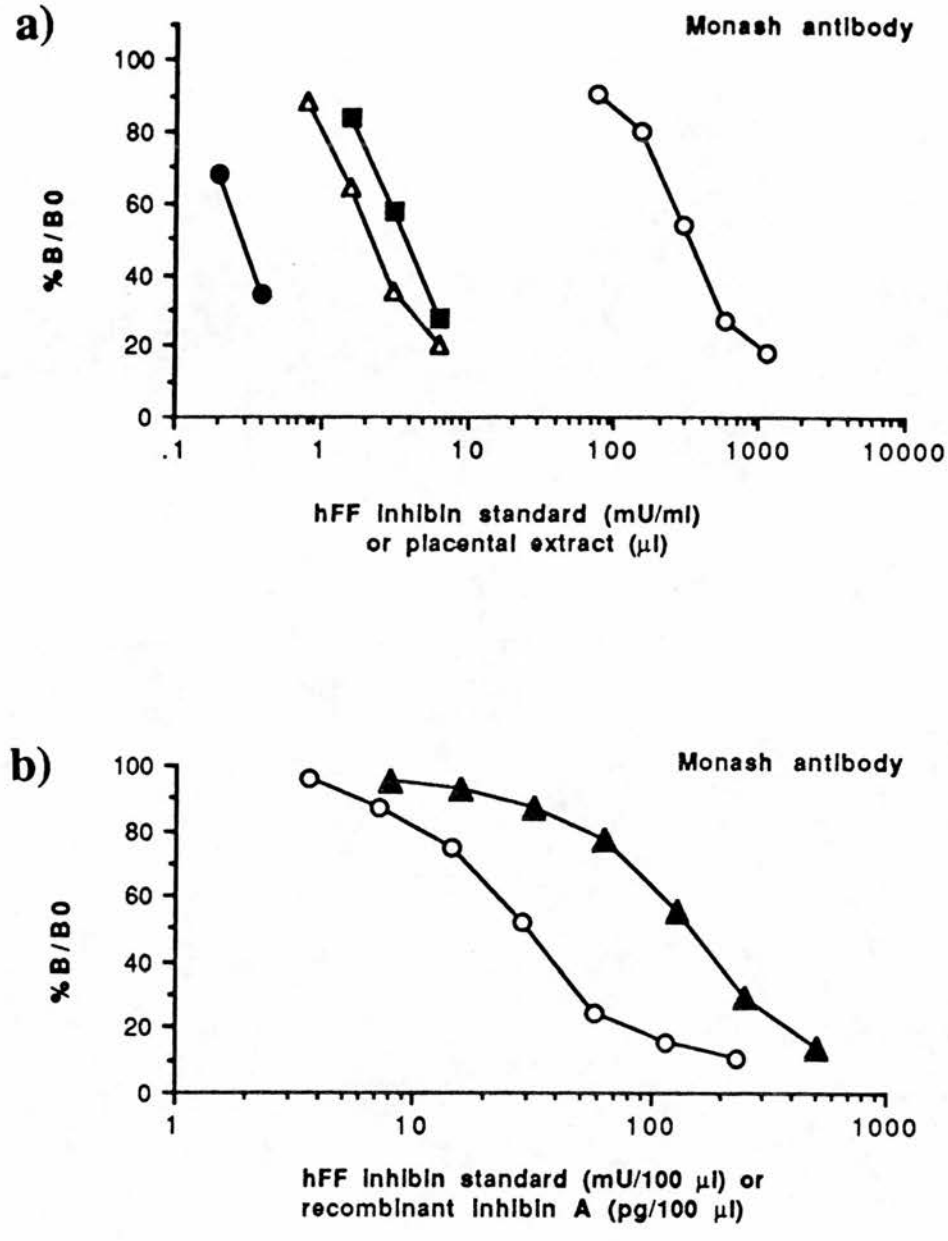

Figure 2.9 (a) Dose-response curves of partially-purified human follicular fluid inhibin standard (O) and placental extracts from 7 weeks (●), 16 weeks (Δ) and delivery (■) demonstrating parallelism. (b) Dilution curves of partially-purified human follicular fluid inhibin standard (O) and human recombinant inhibin A (▲).

fluid. This antibody reveals more immunoactivity of inhibin βA-subunit in placental extracts from various stages of pregnancy than in hFF. These results are difficult to interpret because both inhibin and activin have the same β-subunit structure. It would have been interesting to determine the displacement of recombinant inhibin in the R187 assay system but unfortunately the sensitivity of this system is fairly poor in detecting hFF standards and it was not possible to obtained sufficient recombinant inhibin A for the assay. However, it has already been demonstrated that hFF contains mostly intact inhibin and no activin could be identified (Robertson et al, 1990b). Thus, it is likely that the displacement curves shown by placental extracts in this system represent intact dimeric inhibin.

The immunogenic activity of R187 antibody is not helpful for the RIA of either activin or inhibin but it may have an application for further study of these proteins by immunoblotting analysis and immunocytochemistry. In addition, this type of β-antibody has proved useful in immunocytochemistry (Tovanabutra et al, 1991), immunoblotting analysis of partially-purified proteins (Groome & Lawrence, 1991) and developing a specific immunoassay for inhibin such as the two-site immunoradiometric assay (Knight et al, 1991).

2.3 Characterization of antibodies for Western immunoblotting analysis

2.3.1 Materials & Methods

Antibodies & Proteins

From the data obtained from the experiments in Section 2.2, Y29, R187 and the monoclonal antibody raised against synthetic human inhibin βA-(84-112)-NH_2 (MAb βA) kindly provided by Dr. Nigel Groome (see the details of this antibody in Section 6.3.1.2, Chapter 6) were used to develop a method of Western immunoblotting analysis. 32 kDa inhibin was supplied with the inhibin kit purchased from Monash University. Recombinant inhibin A was kindly donated by Dr. Anthony J. Mason, Genetech Inc., California, USA.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting analysis.

Samples were electrophoresed on precast 5 to 15% sodium dodecyl sulphate (SDS) polyacrylamide slab gels (15 mm thick) under reducing or non-reducing conditions (Laemmli, 1970). Under non-reducing conditions, sample aliquots were heated at 90°C for 5 min with the same volume of sample buffer containing 4% SDS (Bio-Rad Laboratories, Richmond, CA), 20% sucrose (BDH), 0.1% bromophenol blue (Bio-Rad) and 0.375 M Tris-HCl, pH 6.8. For reducing conditions, sample buffer contained 10% 2-mercaptoethanol (BDH) apart from what is mentioned above. Electrode running buffer, pH 8.3 contained 0.025 M Tris base, 0.192 M glycine (BDH) and 0.1% SDS. The samples were electrophoresed at 25 mA and 200 V for 4 h. In order to calibrate molecular weights of proteins, the prestained SDS-PAGE standards, low range (15-110 kDa) were electrophoresed along with the proteins (Bio-Rad). The proteins were electroblotted (semi-dry transferred) on to a nitrocellulose membrane (0.4 micron: Bio-Rad) by using Multiphore II Nova blot electrophoretic transfer unit (Pharmacia LKB). The transfer buffer contained 39 mM glycine, 48 mM Tris base, 0.0375% SDS and 20% methanol. The nitrocellulose membranes were blocked with 5% dried skimmed milk (Marvel) in TBS-0.1% Tween (TBS, Tris-buffered saline, pH 7.4 containing 0.01M Tris-HCl and 0.15M NaCl) for 90 min at room temperature with gentle shaking. The membrane was washed three times, 5 min each, in TBS containing 0.1% Tween and then incubated with primary antibody.

Inhibin α- immunoblotting

The membrane was incubated with Y29 antibody (1:100) in TBS containing 0.1% Tween for 90 min at room temperature with gentle shaking. The membrane was washed with TBS-0.1% Tween three times, 5 min each and then incubated with rabbit anti-sheep immunoglobulin (Dako Ltd, High Wycombe, Bucks) 1:50 in TBS-0.1% Tween for 2 h at room temperature with gentle shaking. The membrane was washed three times with TBS for 5 min each. The membrane was further incubated with sheep-PAP (Dako, Ltd.) 1:100 in TBS for 30 min and washed in TBS 3x 5 min. The membrane was developed in 0.05 M Tris-HCl, pH 7.6 containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 until the bands appeared. The membrane was rinsed with distilled water to stop the reaction and dried at room temperature. The specificity of antibody was checked by replacing the primary antibody with non-immune sheep serum (Vector Laboratories).

Inhibin β-immunoblotting

The membrane was incubated with R187 (1:100) or MAbβA (1:200) antibodies in TBS containing 0.1% Tween for 90 min at room temperture. The membrane was washed with TBS-0.1%Tween 3x 5 min and then incubated with rabbit anti-mouse immunoglobulin (Dako Ltd) 1:50 in TBS-0.1% Tween for 2 h at room temperature with gentle shaking. The membrane was washed again with TBS for 3x 5 min. The membrane was further incubated with Mouse-PAP (Dako Ltd.) 1:100 in TBS for 30 min and washed 3x 5 min in TBS. The membrane was developed as described for inhibin α-immunoblotting. The specificity of the antibody was checked by replacing the primary antibody with non-immune rabbit serum (Dako Ltd).

2.3.2 Results

In Figure 2.10a, the immunoblotting of 0.25 μg bovine inhibin standard with antibody (Y29) directed against human α (1-23) inhibin revealed the 31 kDa band, whereas no band was shown with non-immune sheep serum. Figure 2.10b shows the immunoblotting of recombinant activin A at different concentrations (0.5, 1, 2 and 4 μg), revealing two bands of 28 kDa and 14 kDa with R187 antibody. These bands were not seen with non-immune rabbit serum.

Figure 2.11a shows immunoblotting of 31 kDa bovine inhibin standard (0.25 μg) with Y29 antibody. One band of 31 kDa was demonstrated under non-reducing conditions and one band of 18 kDa under reducing conditions.

Immunoblotting of bovine inhibin standard and recombinant activin A under non-reducing and reducing conditions with MAbβA are shown in Figure 2.11b. For bovine inhibin (0.25 μg), the non-reducing conditions reveal one band at 31 kDa corresponding to intact inhibin while the band at slightly under 16 kDa was shown under reducing conditions. 1 μg of recombinant activin A showed two bands at 28 kDa and 14 kDa under non-reducing conditions and only one thick band of 14 kDa under reducing conditions.

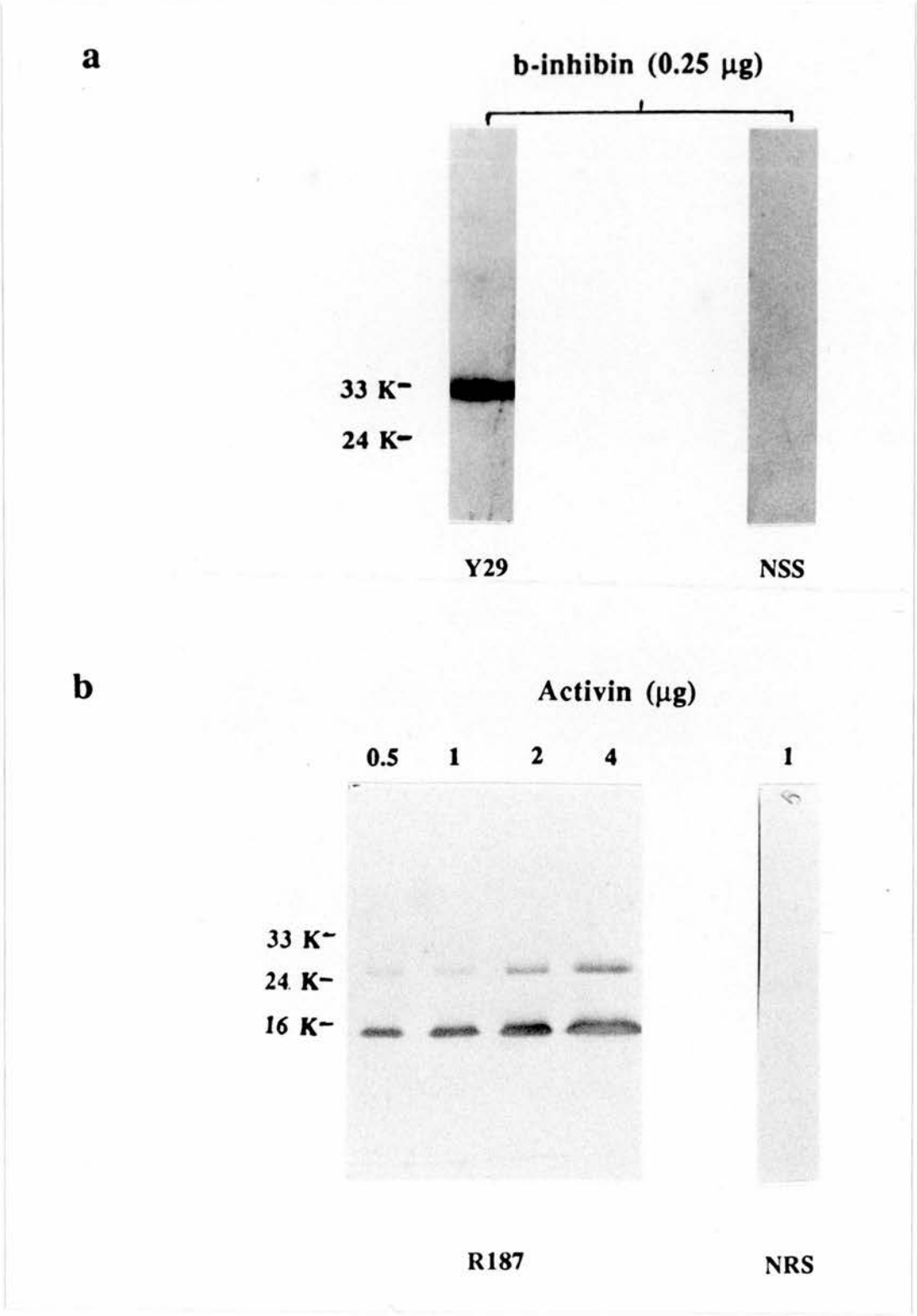

Figure 2.10 (a) Immunoblotting of 31kDa bovine inhibin standard with Y29 antibody and non-immune sheep serum (NSS) under non-reducing conditions. (b) Immunoblotting of recombinant activin A at different concentrations with R187 antibody and non-immune rabbit serum (NRS) under non-reducing conditions.

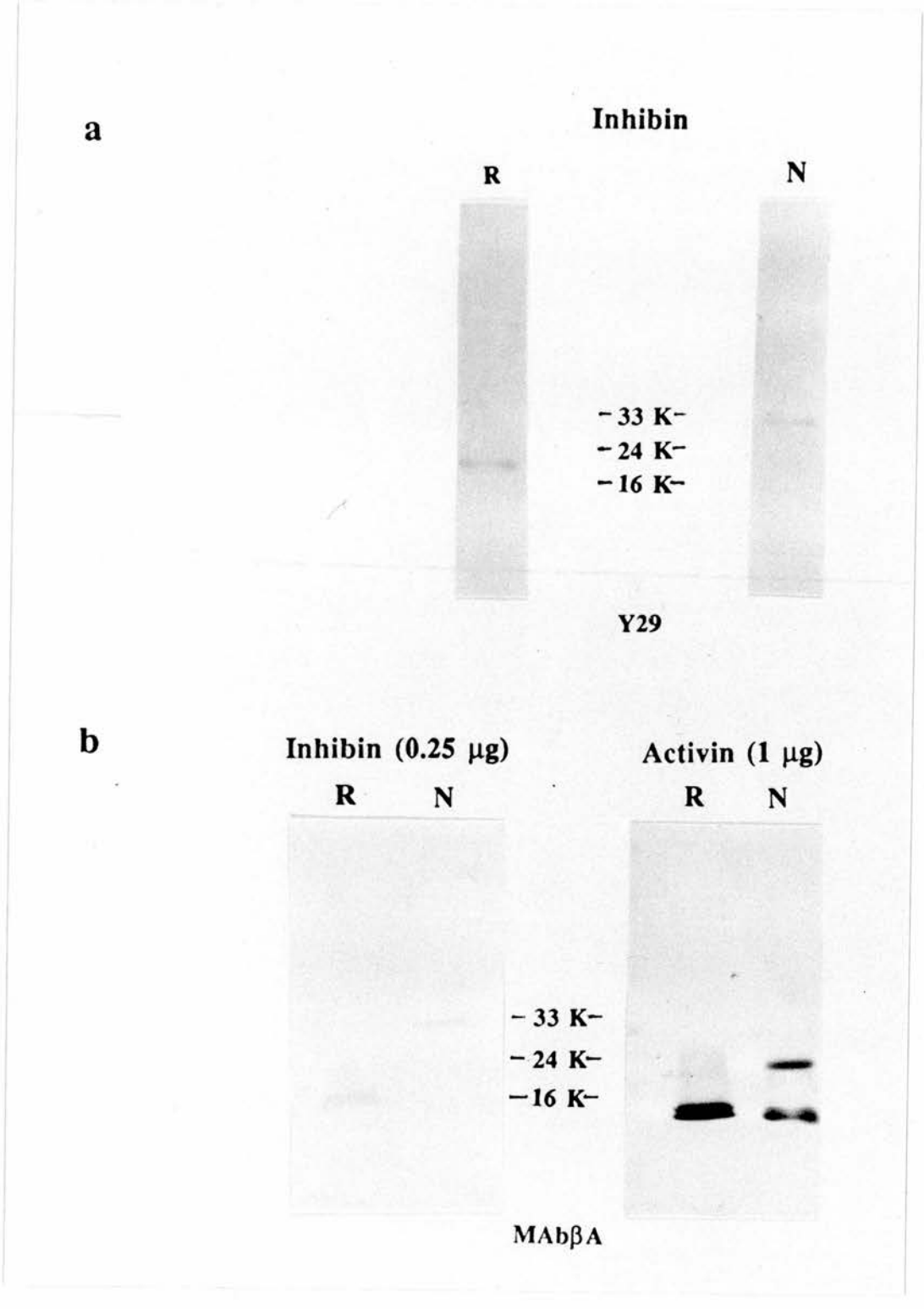

Figure 2.11 (a) Immunoblotting of 31kDa bovine inhibin standard under non-reducing (N) and reducing (R) conditions with Y29 antibody. (b) Immunoblotting of 31kDa bovine inhibin standard and recombinant activin A under non-reducing and reducing conditions with MAbβA antibody.

2.3.3 Discussion

The results from immunoblotting of 31kDa bovine inhibin standard and recombinant activin A using Y29, R187 and MAbβA antibodies demonstrated the specificity of these antibodies to each inhibin subunit. Figure 2.10a shows that, under non-reducing conditions, the Y29 antibody which is directed against the inhibin α-subunit peptide detects intact bovine 31 kDa inhibin. When the intact molecule is broken down into 14 kDa β- and 18 kDa α-subunits under reducing conditions, the Y29 antibody reacts with only the α-subunit suggesting that the specificity of this antibody is limited to the α-subunit only.

The MAbβA monoclonal antibody directed against the inhibin βA-(84-112)-NH_2-subunit detects intact bovine 31 kDa inhibin and 28 kDa activin under non-reducing conditions. This antibody also detects 14 kDa β-subunits which are derived from intact inhibin 31 kDa and activin under reducing conditions. The antibody is specific to the β-subunit only and has proved useful for immunohistochemistry as shown in Chapter 6. The R187 polyclonal antibody which is raised against the inhibin βA-(97-112)-NH_2-subunit also shows specificity for activin under non-reducing conditions and the activin β-subunit under reducing conditions. Surprisingly, immunoblotting of activin with MAbβA and R187 antibodies shows two bands of proteins under non-reducing conditions and one band under reducing conditions. This must be due to the degradation or breaking down of the intact activin molecule into its subunits during storage in acetic acid. This degradation must be taken into consideration for any bioassay experiments examining effects of this activin preparations. However, Y 29 antibody is specific to the inhibin α-subunit, whereas R187 and MAbβA are specific to the inhibin β-subunit. The results from this section and the previous one will be useful for Western blot analysis and immunohistochemistry of inhibin produced by the human placenta.

CHAPTER 3

Relationship between peripheral immunoactive inhibin, human chorionic gonadotrophin, oestradiol and progesterone during human pregnancy

3.1 Introduction

In late human pregnancy, there is an elevation in the peripheral concentration of immunoactive inhibin (McLachlan et al, 1987b; 1987c; Abe et al, 1990; Kettel et al, 1991; Tabei et al, 1991; Smitz et al, 1990; Rombauts et al, 1990) and also bioactive inhibin (Qu et al, 1991). However, reports of the timing and nature of the changes in circulating inhibin vary, particularly in the very early days of pregnancy where the only available data have been obtained following infertility treatment with ovarian superovulation regimes (McLachlan et al, 1987c).

In addition, the source of the circulating inhibin remains uncertain. During the human menstrual cycle, the corpus luteum is the principal source of circulating inhibin (Illingworth et al, 1991) and pharmacologic stimulation of the corpus luteum with hCG at concentrations similar to those seen in early pregnancy cause a marked increase in the circulating inhibin concentration. However, increased inhibin concentrations have also been described during pregnancy in agonadal women who conceived as a result of *in vitro* fertilisation with donor oocytes, thus demonstrating that the corpus luteum cannot be the only source of circulating inhibin at this time. Indeed, the detection of inhibin bioactivity and immunoactivity in placental extracts (McLachlan et al, 1986b; Tovanabutra et al, 1990) including the localization of inhibin subunits (Petraglia et al, 1987a; Merchenthaler et al, 1987; Tovanabutra et al, 1991) and the expression of its mRNA in term placentae (Davis et al, 1987; Reddi et al, 1990b) show that the human placenta is likely to be an important source of inhibin in pregnancy.

In order to investigate the patterns of inhibin secretion in pregnancy and its interaction with other hormones, two studies were performed to obtain serial measurements of the concentration of inhibin, hCG, oestradiol and progesterone in plasma from volunteers 1) prior to the LH surge until day 65 of pregnancy and 2) from 12 weeks of pregnancy until term.

3.2 Materials and Methods

3.2.1 Study design & plasma samples

1) In order to investigate changes in the inhibin concentration around implantation, peripheral blood samples were obtained from four healthy volunteers recruited prior to conception. All subjects were healthy (age range 28-33) with no past history of infertility or endocrine disorders and all conceived within six months of joining the study. Samples were obtained from each subject, three times a week, starting in the late follicular phase (Day 8-10 of the cycle) and continuing either until menstruation or, if conception took place, until 11 weeks after the last menstrual period. In addition, each subject collected daily urine samples throughout the study period and the date of ovulation was taken as the day after the peak urinary LH value as measured by rapid LH immunoassay (Djahanbakhch et al, 1981). One of the subjects conceived during the first study cycle. In the other three subjects, the cycle prior to the conception cycle was taken as the "non-conception" cycle for comparison with the "conception cycle".

2) In order to investigate the changes in inhibin concentration in later pregnancy, peripheral blood samples were obtained from 9 women attending the antenatal clinic at the Simpson Memorial Maternity Pavilion. All subjects were healthy (age range 26-35) with no past history of endocrine disorders. Samples were collected at four-weekly intervals from 12 weeks since the last menstrual period until delivery. In all subjects, the gestation based on the last menstrual period was confirmed by early pregnancy sonography.

Plasma was separated by centrifugation and stored at -20°C for later assay of inhibin, progesterone, oestradiol and hCG.

3.2.2 Radioimmunoassays

1) Inhibin assay

A heterologous radioimmunoassay with antibody to 31kDa bovine (antibody 1989) inhibin and iodinated 31 kDa bovine inhibin as a tracer was used to measure inhibin (Robertson et al, 1988b). A partially-purified human follicular fluid (Reddi et al, 1990a) was used as the standard. Details of the reagents are described in Section 2.2 of Chapter 2.

The assay procedure was also as described in Section 2.2 of Chapter 2, except the sample volume varied from 50 to 200 µl according to the age of pregnancy but the total volume was kept at 200 µl by the addition of pooled inhibin-free postmenopausal plasma (PPS). 200 µl PPS was also added to the standard curve, non-specific binding (NSB) and maximum binding (B_0) tubes to correct for non-specific plasma effects.

2) Progesterone assay

Plasma progesterone was measured using the double antibody RIA developed by Chatterjee, Sweeting, Hillier & Baird (unpublished data).

Reagents:

Assay buffer: 0.25 M citrate buffer, pH 4.0, containing 0.1% gelatin.

Standard: Preg-4-ene 3, 20-dione (Sigma) was prepared in progesterone-free postmenopausal plasma to obtain a working range between 1-127.2 nmol/l.

Antibody: The antibody, raised in a rabbit against progesterone-11α-hemisuccinate conjugated to BSA, was donated by Dr. J. E. T. Corrie, MRC Collaborative Centre, Mill Hill.

Tracer: ^{125}I-progesterone-11α-glucuronide-tyramine was prepared by Mr. Ian Swanston using the chloramine-T technique.

Assay procedure:

50 µl of plasma sample or standard was incubated with 100 µl of antibody (1 in 4,000 in assay buffer), 100 µl of tracer (25,000 cpm/100 µl assay buffer containing 0.036 g % of 8-anilino naphthalene sulphonic acid) for 3 h at room temperature (the non-specific plasma effect was corrected for by adding 50 µl of postmenopausal plasma into the NSB and B_0 tubes). 100 µl of diluted normal rabbit serum (1:640) and 100 µl of diluted donkey anti-rabbit serum (1:20) were added and the assay tubes were incubated overnight at 4°C. After adding 1 ml of 0.9% saline, the assay tubes were centrifuged at 2,500 rpm for 30 min at 4°C. The supernatant was discarded and the precipitate counted. The data was analysed using the Assay Zap programme. The sensitivity of the assay was 3.5 nmol/l whereas the intra- and inter-assay coefficients of variation were 6.2% and 9.1% respectively.

3) Oestradiol assay

Plasma oestradiol was radioimmunoassayed using the ether extraction method described by Glasier et al (1989).

Reagents:

Assay buffer: 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.1% gelatin and 0.01% thiomersal.

Standard: 17β-oestradiol (Sigma) was prepared in assay buffer to obtain the working range of 30 to 10,000 pmol/l.

Antibody: A sheep anti-oestradiol antibody was provided by Dr. R. Webb, IAPGR, Roslin, Midlothian.

Tracer: ^{125} I-oestradiol-3-carboxymethyl ether iodohistamine was prepared by Ms Martha Urquhart using a chloramine-T method.

Assay procedure:

100 µl of sample or standard was pipetted into a 12x75mm borosilicate tube (Sarstedt). The sample was extracted by vortexing with 1 ml diethyl ether (BDH). After freezing the plasma layer in a dry ice/methanol mixture, the ether layer was decanted into a borosilicate tube and dried down under a stream of nitrogen in a heating block. 100 µl of antibody diluted with assay buffer (1:2,000,000) and 200 µl of tracer (12,000 cpm/200 µl assay buffer) were added and the assay tubes incubated at 4°C overnight. The assay tubes were incubated on ice for 15 min and then the antigen-antibody complex was separated from free hormone by adding 500 µl dextran-coated charcoal solution containing 1.25% charcoal (BDH) and 0.125% dextran (BDH). The incubation was continued on ice for a further 15 min. The charcoal was precipitated by centrifugation at 1,500g for 15 min at 4°C. The supernatant was transferred to a polypropylene tube and then counted. The data was analysed as for the progesterone assay. The sensitivity of the assay was 25 pmol/l whereas the intra- and inter-assay coefficients of variation were 8% and 15% respectively.

4) Human chorionic gonadotrophin assay

hCG was assayed using the hCG MAIAclone Monopack purchased from Serono. This assay was developed by combining an immunoradiometric system with a magnetic solid phase. The assay was performed as described in the supplier's instructions.

Reagents:

^{125}I Anti-hCG reagent: Two iodinated mouse monoclonal antibodies to hCG in Tris-buffer containing normal sheep serum, BSA, inert dye (red) and 0.2% sodium azide.

Standards: Standard hCG (1st IRP/3rd IS 75/357) was prepared in horse serum at 5, 10, 25, 100, 250 and 500 mIU/ml.

Assay diluent: Horse serum containing 0.2% sodium azide.

Separation agent: Tris-buffer containing BSA, 0.1% sodium azide and sheep antiserum to fluorescein covalently bound to magnetic particles.
Wash buffer: Tris-buffer containing 0.1% sodium azide.
Assay procedure:
50 µl of sample or standard and 500 µl of ^{125}I anti-hCG were incubated at 37°C for 15 min. 200 µl of separating agent was added to the reaction mixture and the assay tubes were placed on a rack in a magnetic separator for 2 min. The supernatant was carefully decanted and the sediment complex washed with 500 µl of wash buffer to reduce non-specific binding by repeating the separation step in the magnetic separator. The assay tubes were counted and the data processed using the computer programme supplied by Nuclear Enterprises Technology Ltd. The sensitivity of the assay was 5 mIU/ml whereas the intra- and inter-assay coefficients of variation were 3% and 6.5% respectively.

3.2.3 Statistical Analysis

In the calculation of results a log-normal distribution was assumed. Results are, therefore, presented throughout as geometric means with 67% confidence intervals (CI) and all subsequent statistical analysis is carried out on logarithmically-transformed data. The circulating hormone levels in the "conception" and "non-conception" cycles were compared by two-way analysis of variance (ANOVA) using a commercial statistics software package (CLR Anova, Clear Lake Research), taking type of cycle and time since ovulation as within-subject variables.

3.2 Results

Comparison of "conception" and "non-conception" cycles

Figure 3.1 shows the concentrations of inhibin, progesterone, oestradiol and hCG in the first fourteen days of the "conception cycle" and in the preceding "non-conception" cycle. In both cycles, the inhibin concentration (Figure 3.1a) rose after ovulation. In the "non-conception" cycles a peak value was observed on day 8 after the LH peak (geometric mean 239.8 U/l: CI 207.2-277.5 U/l) before the concentrations fell again as luteolysis occurred, reaching 78.8 U/l (CI 53.0-117.1 U/l) on day 14 after the LH peak. In contrast, during the "conception" cycles, the inhibin concentration continued to rise after the mid-luteal phase concentration of 199.9 U/l (CI 173.2-230.6 U/l) to 260.9 U/l (CI 209.3-325.2 U/l) at 14 days after the LH peak. However, when the

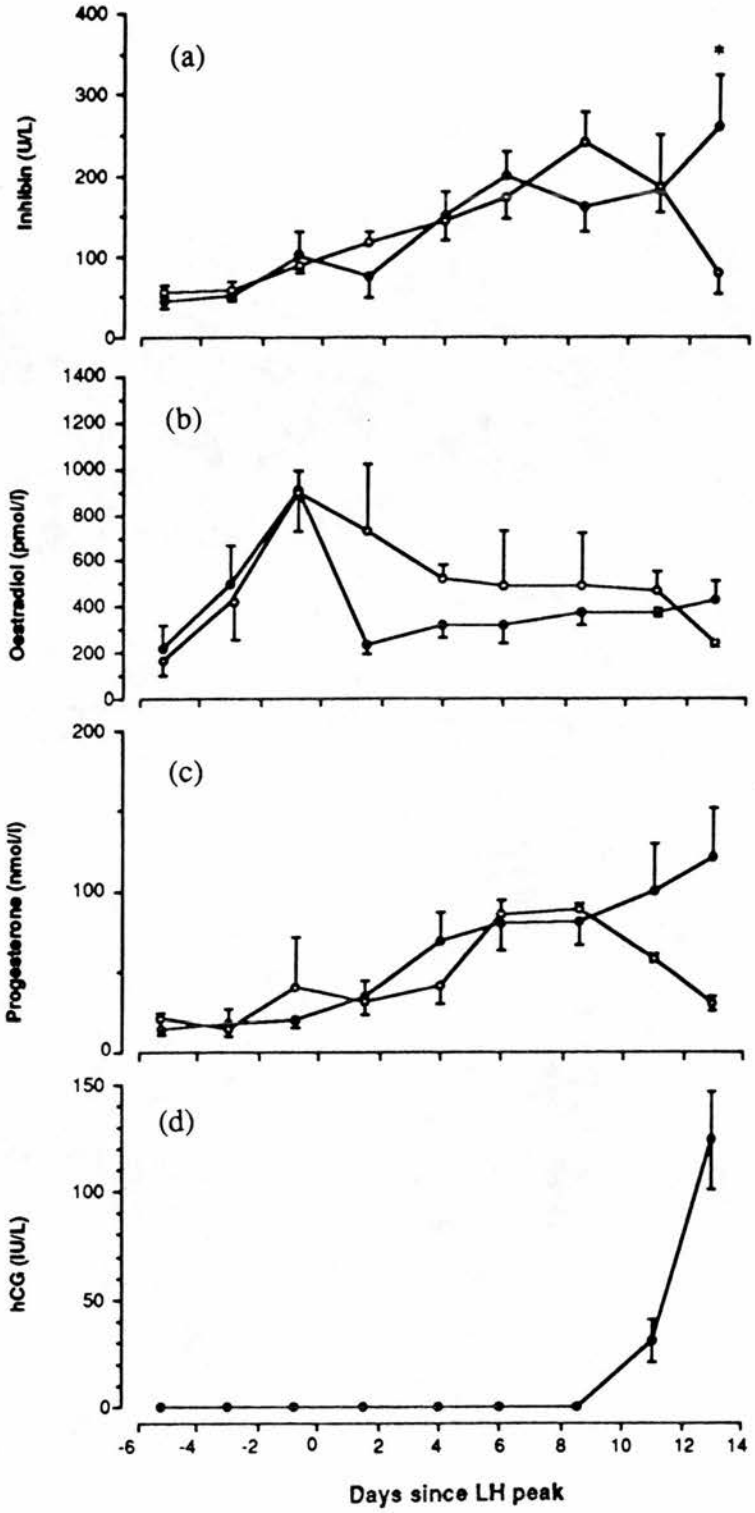

Figure 3.1 Geometric mean (67% confidence intervals) plasma concentrations of inhibin, progesterone, oestradiol and hCG for the three subjects from whom samples were collected during the conception cycle (-●-) and during the previous non-conception cycle (-o-). The data are normalised around the mid-cycle LH peak .

(Conception cycle v. Non-conception cycle; *= $p < 0.05$)

inhibin concentrations in the two cycles were compared using two-way ANOVA, no significant difference was found between the inhibin concentrations in the two cycles. When the inhibin concentrations after the appearance of hCG were compared (days 13-15, Student's paired t test), the inhibin concentrations were found to be significantly ($p<0.05$) greater during the conception cycle. No difference was found in the concentration of either oestradiol or progesterone between the conception cycles and the non-conception cycles before the appearance of hCG (Figures 3.1b and 3.1c).

Serial hormone concentrations throughout pregnancy

The pattern of inhibin secretion in early pregnancy from 4 volunteers is shown in Figure 3.2.

The mean plasma concentrations of inhibin, progesterone, oestradiol and hCG in 4 volunteers from 5 days before the LH peak through conception and implantation until day 65 after the LH peak are shown in Figure 3.3a. The mean plasma concentrations of the same hormones for 9 volunteers between week 12 after the LMP and week 40 after the LMP are shown in Figure 3.3b.

The mean inhibin concentration rose steadily after the LH surge and reached the first peak of 351.9 U/l (CI 275.7-449.0 U/l) around day 16. The level of inhibin declined after the first peak and then rose to reach a second peak (513.0 U/l: CI 442.1-595.3 U/l) around day 47 after the LH surge. The inhibin concentration subsequently fell to a nadir at 16 weeks (227.4 U/l: CI 200.9-257.5 U/l), before rising steadily to a third peak of 614.2 U/l (CI 544.3-693.0 U/l) at term.

The mean plasma concentrations of progesterone, oestradiol and hCG are also shown in Figure 3.3a. Following ovulation, the progesterone concentration rose to reach a first peak between days 13 and 20 (maximum value 126.9 nmol/l: CI 114.5-140.7 nmol/l). A second peak (156.0 nmol/l: CI 130.1-187.2 nmol/l) was then observed at around day 47 of pregnancy, before the progesterone concentration gradually increased from 12 weeks (104.9 nmol/l: CI 96.5-114.2 nmol/l) through term. In contrast to the pattern seen for inhibin and progesterone, the peripheral concentration of oestradiol exhibited a steady rise from the first luteal phase sample (233.2 pmol/l: CI 196.0- 274.4) until term (95,177 pmol/l: CI 85,431-106,034 pmol/l). hCG was first measured above the detection limit of the assay on day 10-11 after the LH surge. The concentration then increased rapidly to reach a peak at day 47 of pregnancy (196,318 IU/L: CI 163,170-236,200 IU/l) before falling to a level of 28,600 IU/l

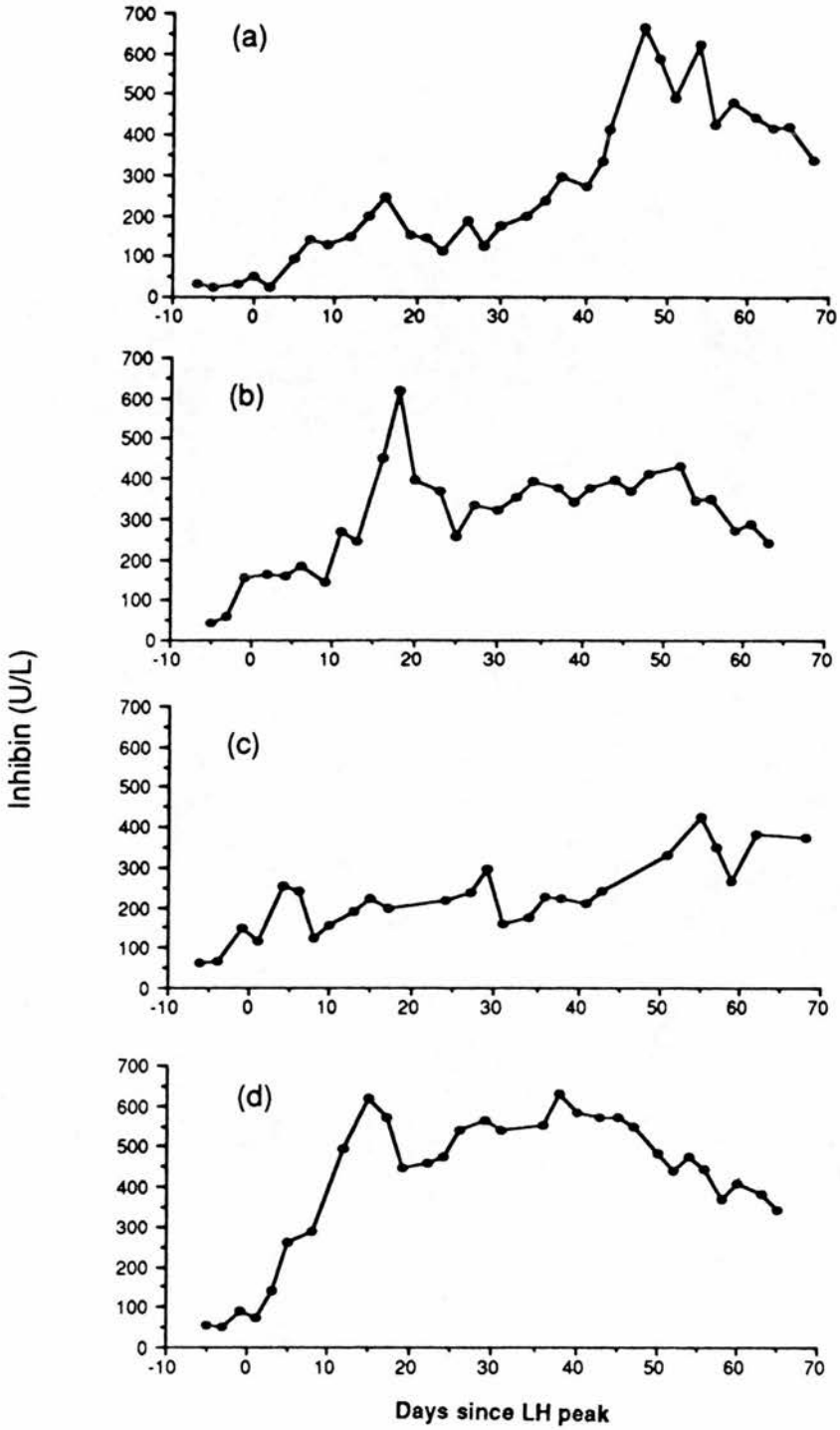

Figure 3.2 Plasma concentrations of inhibin during early pregnancy in 4 volunteers (**a, b, c** and **d**). Data is centred around the day of the LH peak.

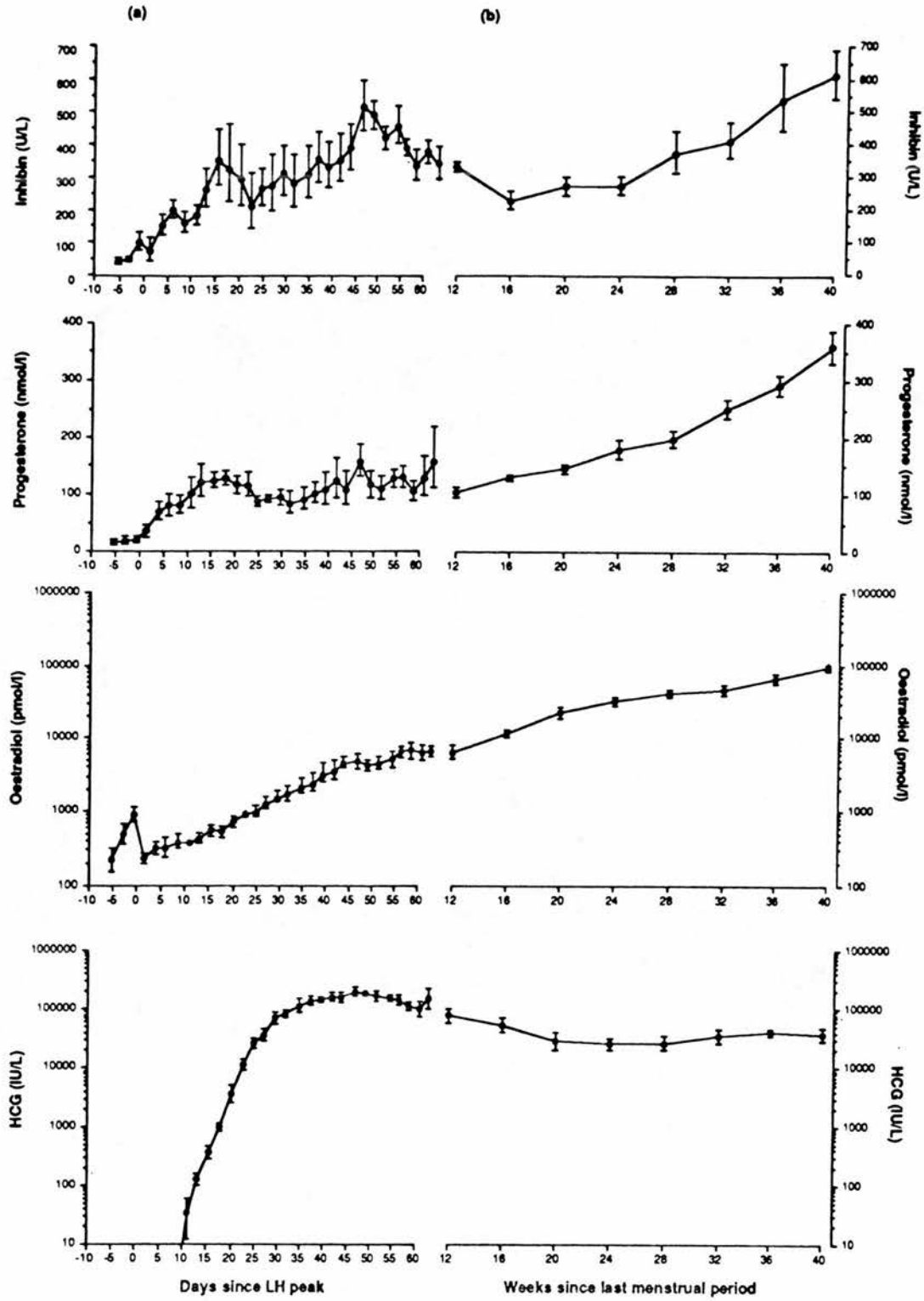

Figure 3.3 Geometric mean (67% confidence intervals) plasma concentrations of inhibin, progesterone, oestradiol and hCG for (a) the four subjects from whom thrice weekly samples were obtained before and after conception and (b) the nine subjects from whom samples were obtained at four-week intervals starting at twelve weeks after the last menstrual period.

(CI 20,400-40,200 IU/l) at 20 weeks. Thereafter, the hCG concentration showed little change for the remainder of the pregnancy.

Correlation between inhibin and hCG, oestradiol and progesterone

It appears from Figures 3.3a and 3.3b that the peaks in mean inhibin concentration coincide with those for progesterone, while the mean concentrations of oestradiol and hCG follow a different pattern. This is reflected in the close correlation between inhibin and progesterone concentrations in both early ($r=0.584$; $p<0.001$) and late pregnancy ($r=0.553$; $p<0.001$). There is also, however, a close correlation between inhibin and oestradiol concentrations in both early ($r=0.602$; $p<0.001$) and late pregnancy ($r=0.361$; $p<0.01$). The correlation between inhibin and hCG was also significant in early pregnancy ($r=0.373$; $p<0.01$) although not in late pregnancy ($r=0.063$; NS). It is notable, however, that the peak concentration of hCG coincided with that for inhibin at day 47 after the LH peak.

3.3 Discussion

This is the first study to report serial measurements of inhibin concentrations in the early days of spontaneously-occurring pregnancy in healthy volunteers. Previous studies of inhibin secretion in pregnancy have commenced later in pregnancy, have not included serial measurements, and have not been well characterised with regard to the timing of conception. The best characterised data previously available is based on inhibin concentrations in pregnancy cycles following superovulation for assisted conception and may not be reflective of the true situation.

It is notable that there are three peaks of inhibin secretion during pregnancy. The first peak is very similar both in magnitude and duration to that previously reported (Illingworth et al, 1990) in non-pregnant volunteers who had received hCG in concentrations similar to those seen in normal human pregnancy. This first peak of inhibin therefore probably reflects corpus luteum activity. The early fall-off in inhibin concentration after the first peak is interesting and raises the possibility that the rescued corpus luteum may exhibit maximum activity at day 16 after ovulation before subsequently reducing its hormone output.

No difference was seen between "conception" and "non-conception" cycles before the appearance of hCG. This finding contrasts with that recently reported in marmosets by Webley et al (1991). It may be that the small number of subjects involved in this study

means that subtle differences between the two cycles have been missed. There is, however, no evidence of differences with the magnitude seen in marmosets.

Do these measurements of immunoactive inhibin reflect increased inhibin bioactivity in terms of an increased inhibitory effect on FSH secretion? It is now clear that the antibody used in this assay binds entirely to epitopes on the inhibin α-subunit and exhibits a marked cross-reaction with either free α-subunit or the pro-αC precursor (Robertson et al, 1989). However, both the putative tissues of origin for inhibin in pregnancy, the corpus luteum and the placenta, have been demonstrated to have positive immunostaining for the inhibin β-subunit (Smith et al, 1992; Tovanabutra et al, 1991). In addition, the presence of either free α-subunit or α-subunit precursors has not yet been established in women. Support for the possibility that this is dimeric inhibin comes from the findings of Qu et al (1991) who reported enhanced inhibin bioactivity in respect of FSH suppression during pregnancy. However, given the methodologic difficulties in adequately removing the high concentrations of sex steroids in pregnancy, the inhibin *in vitro* bioassay may not be specific for non-steroidal FSH suppression under these circumstances.

The physiological function of inhibin in pregnancy remains uncertain. The low concentration of immunoactive FSH during pregnancy may suggest a combined suppressive effect of inhibin and oestradiol on the secretion of pituitary FSH. However, the high concentrations of circulating sex steroids, particularly in late pregnancy, make the significance of the FSH suppressant properties of inhibin uncertain at this time.

A paracrine role has been suggested for inhibin and activin within the placenta. In placental cell cultures, inhibin can reverse the effect of activin on augmenting the stimulation by GnRH of hCG release as well as stimulate progesterone secretion (Petraglia et al, 1989). Inhibin also suppresses the stimulatory effects of GnRH on hCG secretion (Petraglia et al, 1987a). Interestingly, in cultured placental explants, inhibin suppresses hCG secretion by term placenta but not by first trimester placenta (Mersol-Barg et al, 1990). The correlation between peripheral inhibin and hCG which is positive and significant in early pregnancy but not significant in later pregnancy may thus reflect changing paracrine functions of inhibin/activin during pregnancy.

A further possible role for inhibin/activin is in embryogenesis as suggested by the finding of Roberts et al (1991) on the expression of inhibin/activin subunit mRNAs during rat embryogenesis. Activin is a member of the TGF-β group of growth factors

and is a potent mesoderm induction factor (van den Eijnden-Van Raaij et al, 1990) which may be particularly important in promoting axial development in notochord and muscles (Thomsen et al, 1990). Activin is also capable of inhibiting the proliferation of ovarian precursor cell lines as well as the growth of a testicular precursor cell line while inhibin has been found to promote proliferation of ovarian precursor cell lines (Gonzalez-Manchon & Vale, 1990). What is the relationship between the embryogenic functions of predominantly activin and the changes in circulating inhibin concentration seen during pregnancy? This is uncertain as the circulating hormone concentrations are unlikely to be representative of local growth factor activity and further, the activin concentrations in the circulation are unknown.

CHAPTER 4

Placental inhibin immunoactivity and bioactivity during pregnancy and its relation to placental human chorionic gonadotrophin

4.1 Introduction

Placental inhibin bioactivity was first reported in rabbit by Hochberg et al (1981) and in human by Bandivdekar et al (1981). Recently, using the heterologous RIA and the *in vitro* rat pituitary cell bioassay, human term placental extracts were shown to possess inhibin bioactivity and immunoactivity (McLachlan et al, 1986b). Many authors including ourselves (see Chapter 3) have demonstrated an increase in peripheral immunoactive (McLachlan et al, 1987a; Abe et al, 1990; Kettel et al, 1991; Tabei et al, 1991; Smitz et al, 1990; Rombauts et al, 1990) and bioactive inhibin (Qu et al, 1991) during human pregnancy. At present, much more information is required in order to clarify the physiological role of inhibin in pregnancy which still remains unknown.

Furthermore, in recent years an increasing number of proteins have been shown to be secreted by the human placenta. Among these proteins are some known and some unknown growth factors. These factors have effects on cell growth and differentiation (See review by Hill et al, 1987). The differentiation and multiplication of cells were reported to have an effect on their specialized functions e.g hormone production. During the attempt to bioassay inhibin in placental extracts, ovine pituitary cell multiplication caused by unknown growth factors was observed. Term placental extracts had the greatest effect compared to placental extracts from mid-term and 7 weeks of pregnancy (Tovanabutra et al, unpublished data).

The experiments in this chapter were designed: 1) to observe the overall effects of the growth factors in term placental extracts on the FSH secretion of the ovine monolayer pituitary cell cultures in order to exploit the available *in vitro* sheep pituitary cell bioassay to measure inhibin bioactivity in placental extracts. 2) to examine the bioactivity and use *in vitro* immunoneutralization techniques to characterize the bioactive placental inhibin. 3) to observe inhibin immunoactivity in placental extracts and changes in the immunoactivity to bioactivity ratio during pregnancy and 4) to

observe the relationship between placental immunoactive inhibin and hCG content in placental extracts during pregnancy.

4.2 Materials and Methods

Placental extracts

Placental tissue was obtained from pregnancy terminations at 7 weeks, 16 weeks and at full term pregnancy. The placenta was extracted as described in Section 2.2 in Chapter 2. The supernatant was kept at -70° C until the assays were performed.

Inhibin *in vitro* bioassay

Pituitary cell cultures

A sensitive *in vitro* bioassay using sheep pituitary cells was performed as previously described (Tsonis et al, 1986). Briefly, 4-6 mature female sheep pituitary glands were removed and placed in Dulbecco's phosphate-buffered saline (DPBS, Flow Laboratories) containing 7.5 mM glucose. Pituitaries were weighed, chopped into small cubes (3-4 mm), and washed thoroughly in DPBS containing 7.5 mM glucose and 0.1% BSA (Sigma). The chopped tissue was placed in a 25 ml spinner flask with 0.5% trypsin (Type III-S, Sigma) in 12.5 ml DPBS containing 7.5 mM glucose and 0.1% BSA for 30 min at 37°C with gentle stirring. The enzyme solution was replaced with 12.5 ml supplemented Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Rickmansworth, Herts, U.K.), containing 10% (v/v) lamb serum (Flow Laboratories), 2.5% (v/v) fetal bovine serum (Flow Laboratories), 10 mM $NaHCO_3$, 2 mM glutamine (Sigma), penicillin 50 mU/ml and streptomycin 50 mg/ml (Flow Laboratories). The trypsinized tissue was gently stirred for a further 30 min at 37°C. The medium was replaced with 12.5 ml of Ca^{2+} and Mg^{2+} free DPBS ($DPBS^-$; Flow Laboratories) containing 2 mM EDTA and 0.1% BSA and stirred gently for 10 min at 4°C. The tissue fragments were washed 3-4 times with $DPBS^-$ and dispersed using a series of three sterile, siliconized, flame-treated Pasteur pipettes of progressively decreasing bore diameter (5-2 mm). The dispersed cells were transferred into supplemented DMEM, collected by centrifugation at 500 g for 10 min at room temperature and washed twice with supplemented DMEM. The debris and undispersed tissue fragments were removed during the washing step. The cells were resuspended in 20 ml supplemented DMEM and counted using a haemocytometer. Viability, assessed by the trypan blue exclusion test, was accepted from 90% upwards.

Aliquots of 2x 10^5 cells/50 µl were plated in multiple-well plates (Corning Glassware, Corning, NY, U.S.A.). The incubation volume was made up to 600 µl with supplemented DMEM. The plates were incubated in a water-saturated atmosphere of 95% air and 5% CO_2, to maintain the pH of the medium at 7.2-7.4, for 48 h. The medium was then aspirated. The test materials, 100 µl of anti-oestradiol diluted 1:50 in supplemented DMEM and 100 µl of RU486 (a progesterone antagonist) in supplemented DMEM (2ng/100 µl) were added to the wells and then the final incubation volume was made up to 600 µl with supplemented DMEM. The cells were cultured for a further 48 h and the spent media collected and assayed for FSH activity by radioimmunoassay.

Assay procedure

Placental extracts from different stages of pregnancy were charcoal-stripped to remove steroids (McLachlan et al, 1986b) prior to the assay. Five doses of placental extracts were assayed in quadruplicate ranging from 0.05-0.8 µl.

The inhibin reference standard used was a preparation of ovine rete testis fluid (oRTF) with an arbitrary potency of 1 U/mg (Eddie et al, 1979). A charcoal-Sep-Pak-treated ovine follicular fluid (CGT-1083-oFF) was used as the quality control. They were assayed in quadruplicate at five doses ranging between 12.5-200 mU/well for standard and 0.823-66.67 nl/well for quality control. The FSH dose-response curves of standard and samples were expressed as a percent inhibition of control wells with no exposure to inhibin. Regression analysis was calculated using a computer programme (Scott et al, 1980). The slope, precision index and significance of regression (Finney's G) were calculated (Finney, 1964). The relative potencies of samples showing parallellism and linearity to the reference standard ($p>0.05$: Borth, 1976) were calculated by comparison with the reference standard. The sensitivity of the assay was 0.05-0.1 U/ml, the index of precision 5.2% and the significance of regression ($G)<0.02$. The inter-assay coefficient of variation was 14.9%.

Neutralization of inhibin bioactivity

Different doses of charcoal-stripped placental extracts, human follicular fluid (hFF) and ovine follicular fluid were assayed to produce an FSH dose-inhibition curve and doses producing about 50% inhibition were chosen to study the *in vitro* neutralization effect of the Y29 polyclonal antibody. The test materials at selected doses were incubated with the antibody at different concentrations at room temperature for 2 h before being added to the preincubated pituitary cell cultures in triplicate. After making

up the final incubation volume to 600 µl with supplemented DMEM, the culture plates were incubated for a further 48 h and the spent media were collected and assayed for FSH.

Radioimmunoassays

1) Ovine FSH assay

FSH in culture media was measured using a specific double-antibody radioimmunoassay as previously described (McNeilly et al, 1976).

Reagents:

Assay buffer: 0.075 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1% bovine serum albumin (RIA grade, Sigma) and 0.01% thiomersal (BDH).

Standards: Ovine FSH (NIH-oFSH-S9) was diluted in assay buffer to obtain the working range from 15.6 to 1000 ng/ml.

Antiserum: Anti-human FSH sera, M91/1 (Lynch & Shirley, 1975) was diluted to 1 in 5,000 with 0.075 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.05 M EDTA and 0.6% normal rabbit serum (SAPU)

Tracer: The ^{125}I-NIAMDD-oFSH-1 was prepared using the lactoperoxidase method as previously described (McNeilly et al, 1986).

Assay procedure:

The assay was performed by incubating 100 µl standards or 50 µl of samples, 100 µl of the diluted antibody and 300 or 350 µl of assay buffer at 4°C overnight. 100 µl of ^{125}I- labelled antigen (15,000 cpm/100µl in assay buffer) was added and the incubation continued at 4°C for a further 24 h. The antigen-antibody complexes were precipitated by adding 100 µl of donkey anti-rabbit serum (SAPU) diluted to 1 in 20 with 0.075 M phosphate buffer, pH 7.4, containing 4% dextran (BDH). After 18 h incubation at 4°C, 0.75 ml of 5% PEG in 0.075 phosphate buffer containing 0.015 M NaCl was added and the assay tubes centrifuged at 1,600g for 20 min at 4°C. The supernatant was aspirated and the precipitate counted in a gamma counter (Pharmacia-Wallac 1261 multigamma manual gamma counter). The data were analysed using the 'Assay Zap Universal Assay Calculator'. The sensitivity of the assay was 16 ng/ml and the coefficients of variation of inter-assay and intra-assay were 6.6% and 9.9% respectively.

2) Ovine LH assay

Culture media LH was assayed using a double-antibody RIA as previously described (McNeilly et al, 1986).

Reagents:

Assay buffer: 0.075 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1% bovine serum albumin (RIA grade, Sigma) and 0.01% thiomersal (BDH).

Standards: Ovine LH (NIH-oLH-S18) was diluted in assay buffer to obtain the working range from 0.1 to 20 ng/ml.

Antiserum: Anti-ovine LH sera, R29, was diluted to 1 in 120,000 with 0.075 M phosphate buffer (pH 7.4) containing 0.15 M NaCl.

Tracer: LH preparation (LHM4)was iodinated using a lactoperoxidase method by Mrs Gwen Cowen.

Assay procedure:

The assay was performed by incubating 100 µl standards or samples, 100 µl of the diluted antibody and 200 µl of assay buffer at 4°C overnight. 100 µl of ^{125}I- labelled antigen (15,000 cpm/100µl in assay buffer) was added and the incubation continued at 4°C for a further 24 h. The antigen-antibody complexes were precipitated by adding 100 µl of donkey anti-rabbit serum (SAPU) diluted to 1 in 32 with assay buffer and 100 µl of normal rabbit serum diluted 1:800 with assay buffer. The incubation was continued for a further 18 h at 4°C and 1ml of 0.9% NaCl was added. The assay tubes were centrifuged at 1,600g for 20 min at 4°C. The supernatant was decanted and the precipitate counted in a gamma counter. The data were analysed using the 'Assay Zap Universal Assay Calculator'. The sensitivity of the assay was 0.2 ng/ml and the coefficients of variation of intra-assay and inter-assay were 4.9% and 14% respectively.

3) Ovine Prolactin (PRL) assay

Ovine prolactin was assayed using a double-antibody RIA as previously described (McNeilly & Andrews, 1974).

Reagents:

Assay buffer: 0.075 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1% bovine serum albumin (RIA grade, Sigma) and 0.01% thiomersal (BDH).

Standards: Ovine PRL (NIH-oPRL-S15) was diluted in assay buffer to obtain the working range from 0.8 to 200 ng/ml.

Antiserum: Anti-ovine PRL sera, R2532, was diluted to 1 in 128,000 with 0.075 M phosphate buffer (pH 7.4) containing 0.15 M NaCl.

Tracer: The ^{125}I-NIAMDD-oPRL-17 was prepared using a lactoperoxidase method as previously described (McNeilly et al, 1986)

Assay procedure:

The assay was performed by incubating 30 µl standards or samples, 100 µl of the diluted antibody, 100 µl of ^{125}I- labelled antigen (15,000 cpm/100µl in assay buffer) and 100 µl of assay buffer at 4°C for 24 h. The antigen-antibody complexes were precipitated by adding 100 µl of donkey anti-rabbit serum (SAPU) diluted 1 in 32 with assay buffer and 100 µl of normal rabbit serum diluted 1:400 with assay buffer. The incubation was continued for a further 24 h at 4°C and 1ml of 0.9% saline was added. The assay tubes were centrifuged at 1,600g for 25 min at 4°C. The supernatant was decanted and the precipitate counted in a gamma counter. The data were analysed using the 'Assay Zap Universal Assay Calculator'. The sensitivity of the assay was 0.8 ng/ml and the coefficients of variation of intra-assay and inter-assay were 7% and 9% respectively.

4) Inhibin assay

Inhibin immunoactivity in placental extracts was measured using the heterologous RIA with the Monash antibody as described in Section 2.2 in Chapter 2.

5) Human chorionic gonadotrophin assay

hCG was assayed using the hCG MAIA clone Monopack purchased from Serono. The assay was performed as described in Section 3.2 in Chapter 3.

Cell counting

Pituitary cells were removed from the culture wells by trypsinization (modified method from Sigma Chemical Co. Catalogue). Briefly, after the conditioned medium was collected, the cells were washed with 400 µl of DPBS$^-$ and then incubated with 200 µl of 0.2% trypsin in DPBS$^-$ containing 0.08% EDTA for 20 min at 37°C. The trypsin activity was neutralized by adding 600 µl of supplemented DMEM before the solution was transferred into a sterile conical tube. The cells were collected by spinning at 500g for 10 min at 20°C and then resuspended in 400 µl of supplemented DMEM. Cells were counted using a haemocytometer.

Statistical analysis

For any comparison the data were subjected to one-way analysis of variance and subsequently examined using Newman-Keul's test (ANOVA; Clear Lake Research, Houston, Texas, U.S.A.).

4.3 Results

4.3.1 Characterization of sheep pituitary cells inhibin bioassay

Mitogenic activity in term placental extracts was observed in sheep pituitary cell cultures during the inhibin bioassay. As shown in Figure 4.1, after the incubation with different doses of term placental extract for 48 h the number of pituitary cells increased from $2.00 \pm 0.22 \times 10^5$ (control; supplemented DMEM) to $3.01 \pm 0.03 \times 10^5$ (0.1 µl placental extract) ; $p<0.01$. The cell number did not significantly change at the doses between 0.1 to 1 µl of placental extract. At the higher doses of placental extract the number of cells increased from $2.00 \pm 0.22 \times 10^5$ (control) to $3.84 \pm 0.12 \times 10^5$ (1µl placental extract); $p<0.01$ and $6.58 \pm 0.19 \times 10^5$ (50 µl placental extract); $p<0.01$.

Conditioned medium was assayed for ovine FSH, LH and PRL. The release of ovine LH and PRL was not different in the presence or absence (control) of different doses of placental extract as shown in Figures 4.2 and 4.3. In Figure 4.4, on the contrary, FSH release was suppressed in a dose-dependent manner by placental extract (0.20-1.56 µl) despite an unchanged cell number as shown in Figure 4.1.

4.3.2 Inhibin bioactivity in placental extracts from different stages of pregnancy

FSH dose-response curves of placental extracts from 7 weeks, 16 weeks and term pregnancy are parallel to the inhibin reference standard (oRTF) as shown in Figure 4.5. The bioactivity of inhibin in charcoal-stripped placental extracts from various stages of pregnancy was measured. The level remained unchanged from 7 weeks (73.68 ± 15.90 U/g, n=5) through 16 weeks (87.67 ± 5.96 U/g, n=5) until term (91.7 ± 7.58 U/g, n=5) as shown in Figure 4.6.

4.3.3 Immunoneutralization of placental inhibin using Y29 and MAbβA antibodies and the effect of activin on FSH secretion in sheep pituitary cell cultures

Inhibin activity in placental extracts from different stages of pregnancy and hFF generated FSH inhibition-curves in a dose-related manner, as shown in Figure 4.7a. The dose of each inhibin preparation (6.25 µl of hFF and 3.125 µl of placental extracts from different stages of pregnancy) producing suppression of FSH release at around 50% of control was chosen for *in vitro* immunoneutralization by the Y29 antibody. In Figure 4.7b an increase in volume of the antibody from 12.5 µl could neutralize the

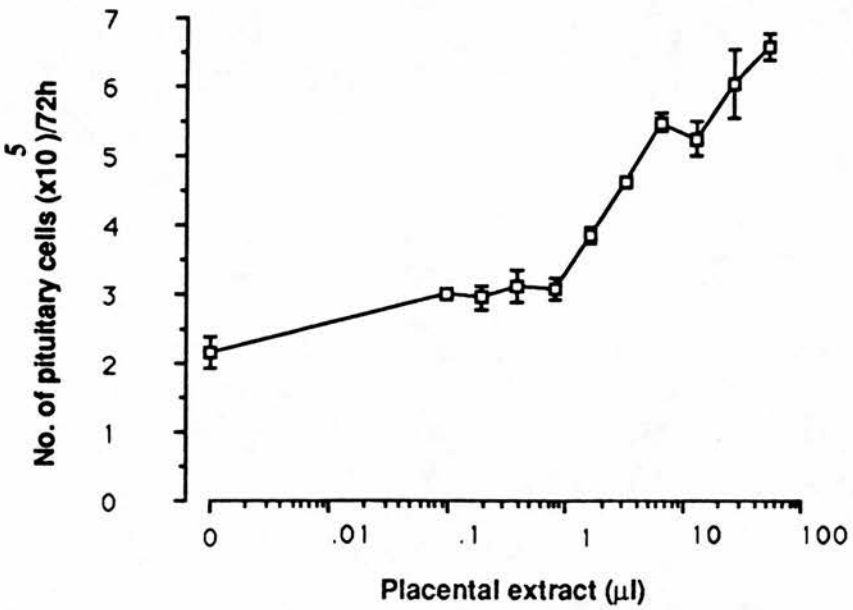

Figure 4.1 Effect of term placental extract on the proliferation of sheep pituitary cell cultures for 48h, after an initial 48 h preincubation with no treatment. Values represent the mean ± SEM of quadruplicate wells/dose.

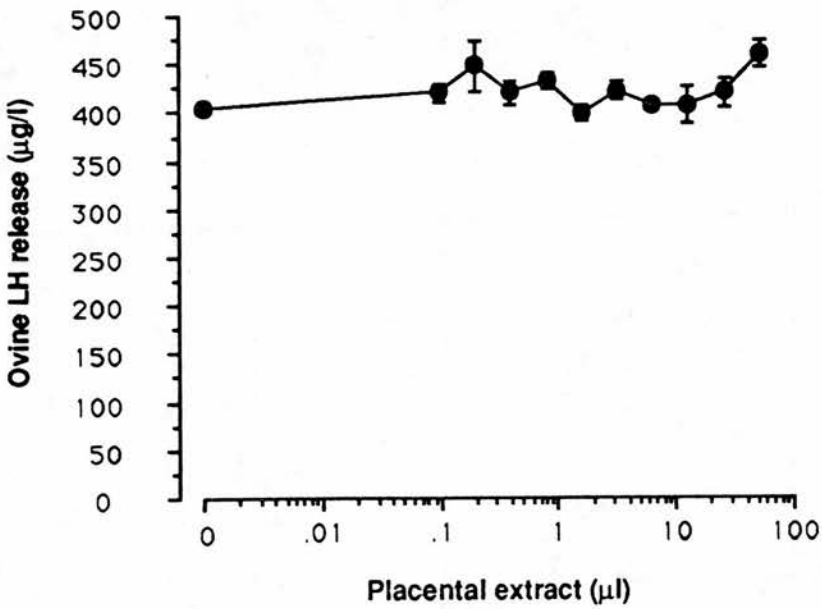

Figure 4.2 Effect of term placental extract on the release of LH from sheep pituitary cell cultures for 48h, after an initial 48 h preincubation with no treatment. Values represent the mean ± SEM of quadruplicate wells/dose.

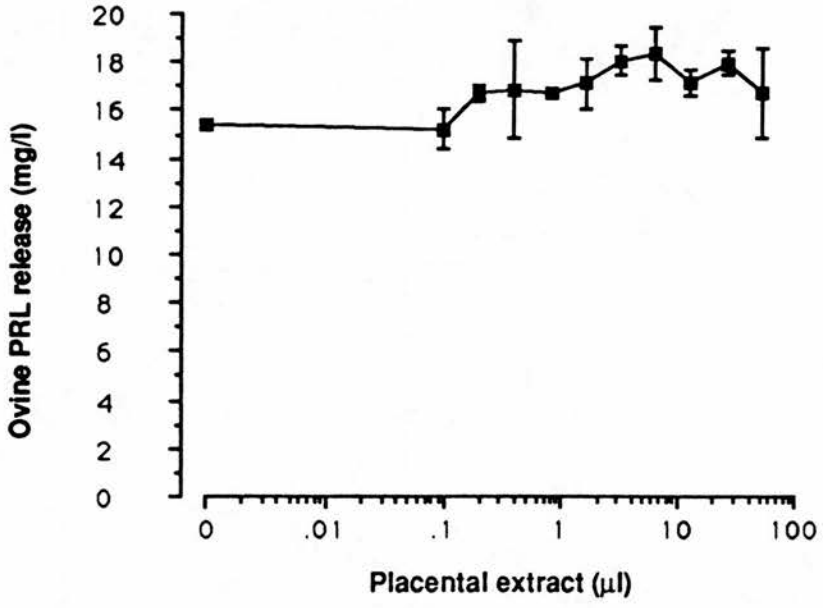

Figure 4.3 Effect of term placental extract on the release of prolactin from sheep pituitary cell cultures for 48h, after an initial 48 h preincubation with no treatment. Values represent the mean ± SEM of quadruplicate wells/dose.

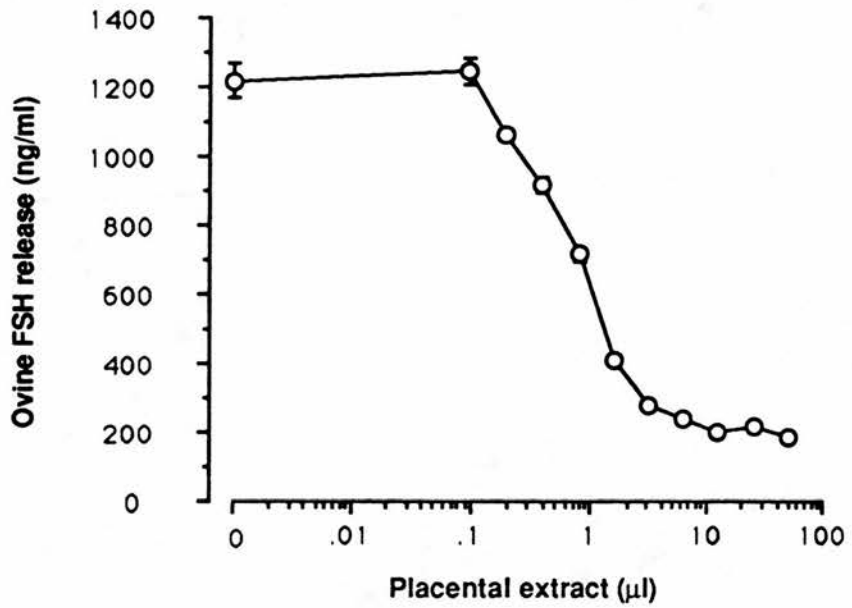

Figure 4.4 Effect of term placental extract on the release of FSH from pituitary cell cultured for 48h, after an initial 48 h preincubation with no treatments. Values represent the mean ± SEM of quadruplicate wells/dose.

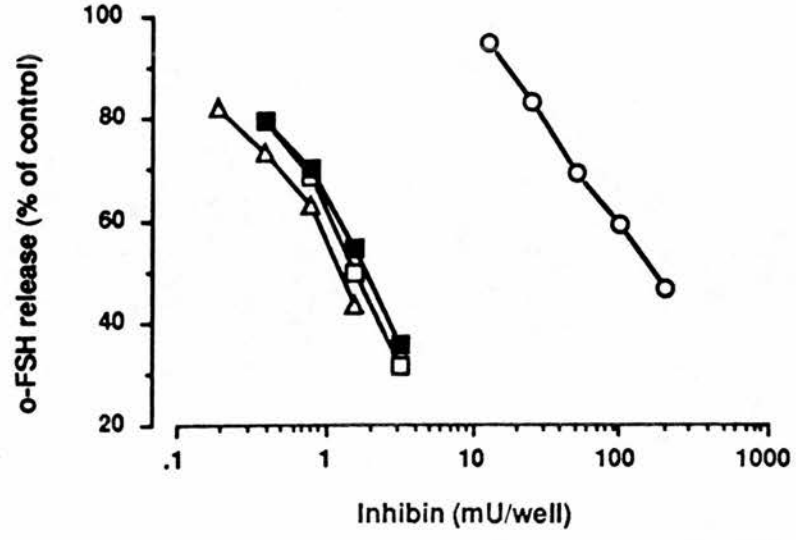

Figure 4.5 The FSH dose-response curves of oRTF [inhibin reference standard] (O) and placental extracts at 7 weeks (Δ), 16 weeks (□) and term (■) demonstrating parallelism in the bioassay.

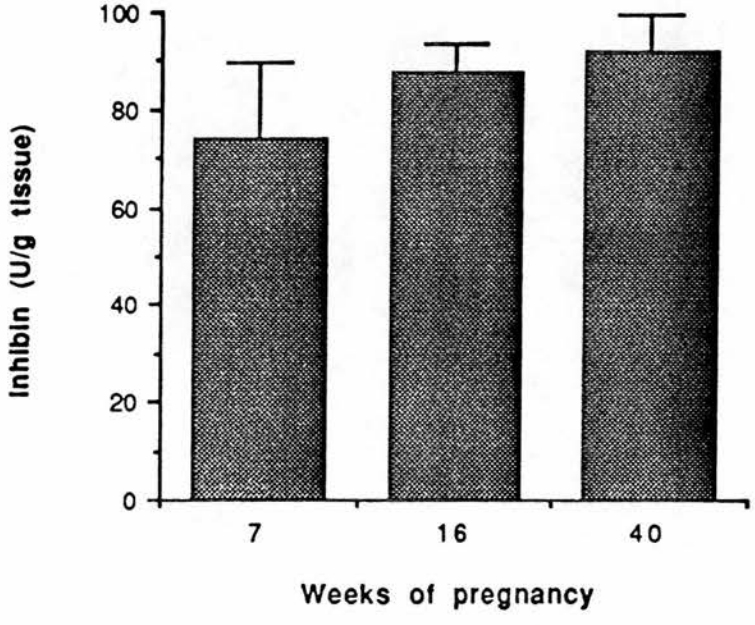

Figure 4.6 Bioactive inhibin levels in placental extracts from 7 weeks, 16 weeks and term pregnancy. Values represent the mean ± SEM.

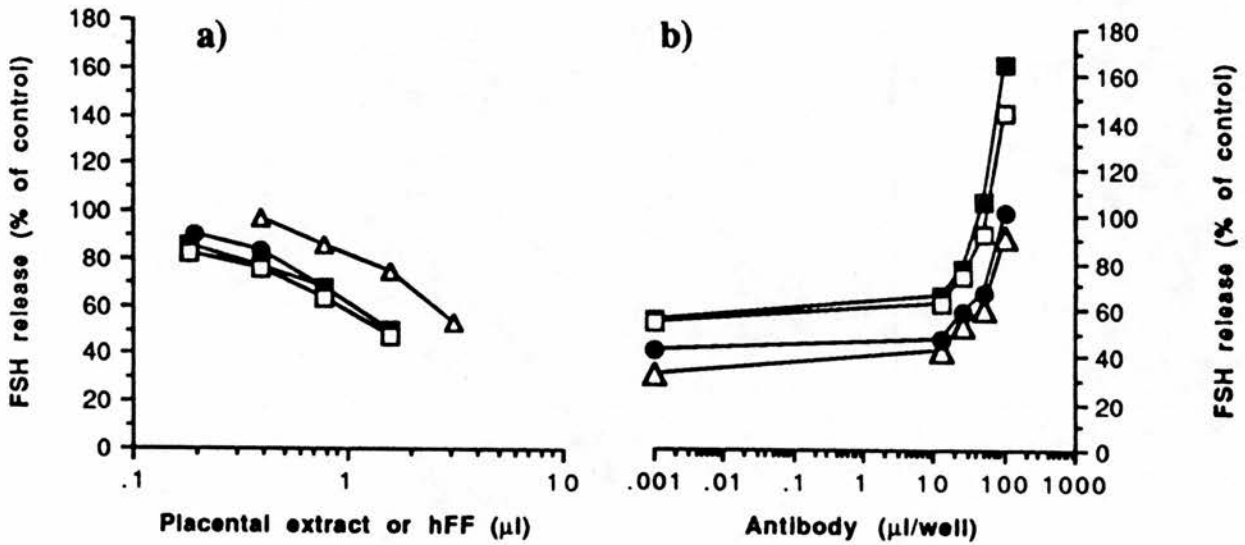

Figure 4.7 (a) The suppressing activity of bioactive inhibin in hFF (Δ) and placental extracts from 7 weeks (●), 16 weeks (■) and term pregnancy (□) on FSH secretion from sheep pituitary cell cultures. (b) The reverse effect of inhibin on FSH release by immunoneutralization using Y29 antibody.

inhibin bioactivity resulting in an increase in oFSH release. The inhibin activity in each preparation was completely neutralized when the volume of the antibody was increased to 100 μl. Non-immune sheep serum had no neutralization effect in control wells.

For mid-term and term placental extracts a stimulatory effect on FSH release was observed after neutralization by the antibody as shown in Figure 4.8a. This stimulatory effect in mid-term and term placental extracts could increase FSH release to 165.35 ± 31.57% and 144.72 ± 25.55% respectively of the control level. A stimulatory effect of recombinant activin A was also observed in sheep pituitary cell cultures. The pattern of stimulation was dose-dependent; FSH release was increased up to 140 ± 5.45% of control at 1 μg of activin/well (Figure 4.8b).

Figure 4.9 demonstrates immunoneutralization of hFF and placental extracts from 7 weeks, 16 weeks and term pregnancy by the MAbβA antibody. The suppression of FSH release (% of control) by the inhibin in each preparation (6.25 μl of hFF and 3.125 μl of placental extracts from different stages of pregnancy) did not change statistically, even at the highest concentration (10 μg of immunoglobulin).

4.3.4 Inhibin immunoactivity in placental extracts from different stages of pregnancy

The levels of immunoactive inhibin in placental extracts from 7 weeks, 16 weeks and term are shown in Figure 4.10. The activity decreased from 84.90 ± 7.15 U/g at 7 weeks through 19.87 ± 4.49 U/g at 16 weeks ($p<0.01$) to 5.94 ± 0.55 U/g at term ($p<0.01$).

4.3.5 hCG immunoactivity in placental extracts from different stages of pregnancy

The levels of immunoactive hCG in placental extracts from 7 weeks, 16 weeks and term are shown in Figure 4.11. The hormone content decreased from 5559.24 ± 1887.88 IU/g at 7 weeks through 181.12 ± 57.28 U/g at 16 weeks ($p<0.01$) to 15.68 ± 3.99 U/g at term ($p<0.01$).

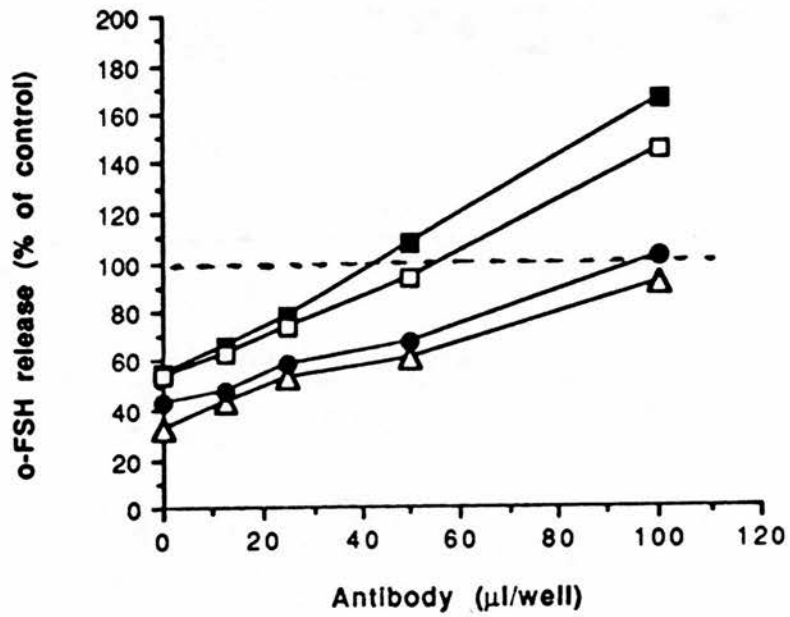

Figure 4.8 (a) The reverse effect of inhibin bioactivity, by immunoneutralization using Y29 antibody, in hFF (●) and placental extracts from 7 weeks (Δ), 16 weeks (□) and term pregnancy (■) on FSH secretion from sheep pituitary cell cultures.

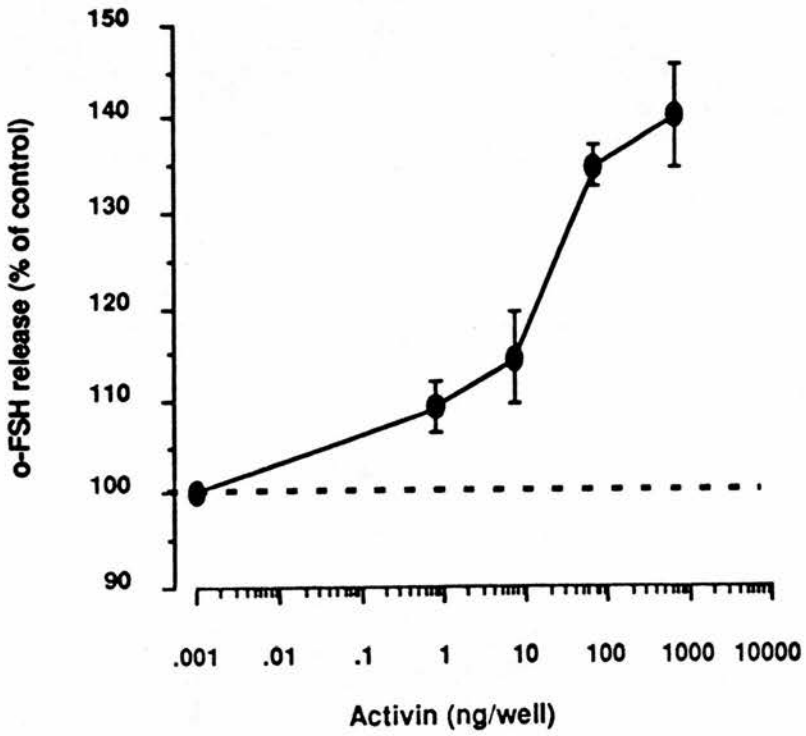

Figure 4.8 (b) Effect of recombinant activin A on FSH release from sheep pituitary cell cultures. Values represent the mean ± SEM of quadruplicate wells/dose.

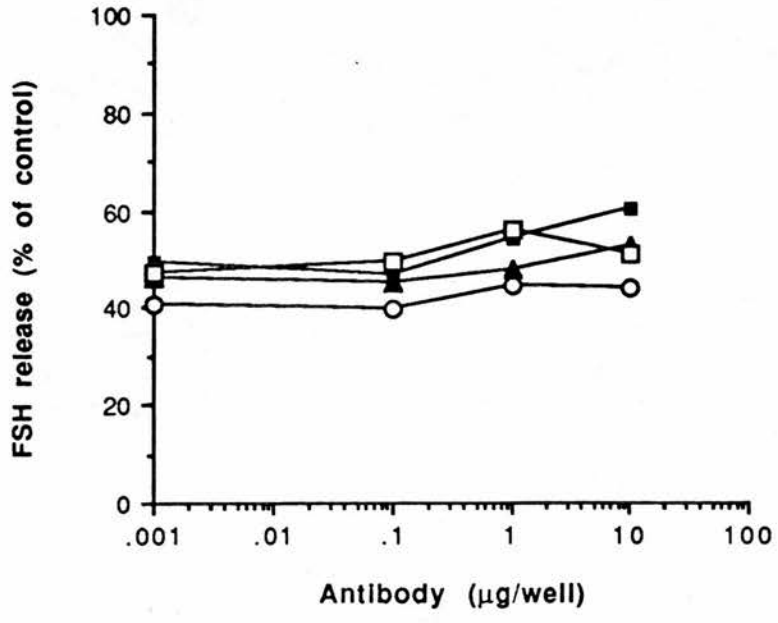

Figure 4.9 Effect of MAbβA antibody on the suppressing activity of bioactive inhibin in hFF (O) and placental extracts from 7 weeks (■), 16 weeks (▲) and term pregnancy (□) on the FSH secretion from sheep pituitary cell cultures.

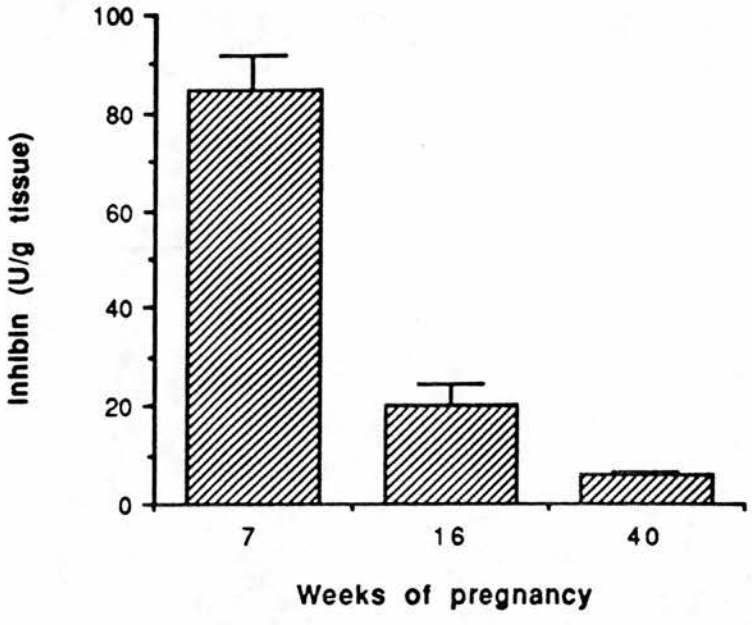

Figure 4.10 Immunoactive inhibin levels in placental extracts from 7 weeks, 16 weeks and term pregnancy. Values represent the mean ± SEM.

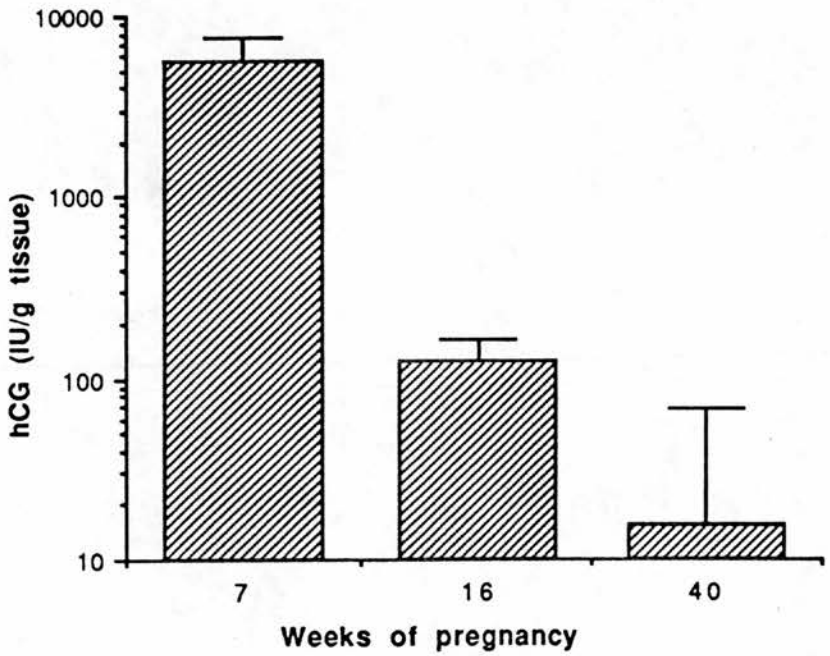

Figure 4.11 Immunoactive hCG levels in placental extracts from 7 weeks, 16 weeks and term pregnancy. Values represent the mean ± SEM.

4.4 Discussion

The bioactivity of inhibin in placental extracts from different stages of pregnancy was examined using the sheep pituitary cell culture bioassay. Since there is direct and indirect evidence for the existence of different growth factors in the human placenta (Hill et al, 1987), the mitogenic activity of human placental extracts was anticipated during the bioassay. This anticipation was confirmed by the finding from our preliminary study that 7 weeks, 16 weeks and term placental extracts caused sheep pituitary cell multiplication *in vitro* and the mitogenic activity was highest in term placental extracts. The present data show that term placental extract possessed high mitogenic activity which caused cell numbers to increase several fold (Figure 4.1). The production of hormones and other specialized functions are influenced by the degree of cellular differentiation. In contrast, the results from this chapter demonstrate that the release of ovine LH and PRL during pituitary cell multiplication caused by placental mitogenic factors was not altered (Figures 4.2 and 4.3). The suppression of ovine FSH release by bioactive inhibin in placental extracts has the same dose-dependent pattern as other inhibin preparations (Figure 4.5). This finding is similar to that reported by Tsonis et al (1988b) who found that pituitary cell division and multiplication had no effect on the bioassay of inhibin in conditioned medium from human granulosa cells using sheep pituitary cell cultures. The mitogenic activity of placental extracts does not interfere with the measurement of bioactive inhibin in placental extracts. A possible explanation of the unchanged hormone production could be that most pituitary cells are not gonadotrophs, some of them are lactotrophs or fibroblasts and the cell type that increases in number has not been identified whether they are gonadotrophs or not. The identity and physiological mechanism of this placental mitogenic factor needs further investigation. However, the placental mitogenic factor could be a combination of different growth factors which are abundant in placentae. These unknown growth factors are more likely to be proteins, due to the removal of steroids from placental extracts by charcoal-stripping prior to the assay.

The FSH-suppressing activity in placental extracts appears to be mostly due to inhibin because of the complete abolition of the inhibitory effect after immunoneutralization. In addition, the bioactivity of inhibin which can be immunoneutralized *in vitro* by Y29 antibody (anti 1-23 α-subunit) but not by MAbβA antibody (anti β-subunit) showed that the blocking of the N-terminal fragment of the α-subunit has an effect on the function of the bioactive site of the inhibin molecule. This is supported by previous studies which have shown that anti-p1-26 α-inhibin can immunoneutralize sheep

ovarian inhibin bioactivity *in vitro* and *in vivo* (Mann et al, 1989); anti-human 1-32 α-inhibin can immunoneutralize inhibin bioactivity *in vivo* (Mersol-Barg et al, 1990) and anti-human 6-30 α inhibin can immunoneutralize human ovarian inhibin bioactivity in rat pituitary cell cultures (Sinosich et al, 1991).

The stimulatory effect of mid-term and term placental extracts on FSH secretion (Figure 4.8a) which is similar to the stimulatory effect of activin on FSH secretion (Figure 4.8b), suggests the production of activin by the human placenta in later stages of pregnancy. Furthermore, the bioactivity of inhibin at later stages of pregnancy might be the combined effects of inhibin and activin.

Inhibin bioactivity in placental extracts does not change throughout pregnancy (Figure 4.6) whereas inhibin immunoactivity decreases towards term (Figure 4.10). Taken together, the indirect evidence of the presence of an activin-like effect in placental extracts and the fact that the heterologous radioimmunoassay using the Monash antibody has been shown to measure various immunoactive forms of inhibin, some of which, such as pro-αC, have no bioactivity, suggest that at various stages of pregnancy the placenta produces different amounts and molecular species of inhibin or activin.

hCG immunoactivity in placental extracts decreases towards term (Figure 4.11) in the same manner as inhibin immunoactivity. This suggests a relationship between hCG and inhibin which are both synthesized by syncytiotrophoblast cells. This is supported by the paracrine function of inhibin in the regulation of hCG secretion in placental cell cultures (Petraglia et al, 1987a) and the suppressive effect of inhibin on hCG secretion in placental explant cultures (Mersol-Barg et al, 1990).

CHAPTER 5

Inhibin immunoactivity and bioactivity in amniotic fluid during pregnancy

5.1 Introduction

Inhibin bioactivity and immunoactivity have been demonstrated in human placental extracts (McLachlan et al, 1986b) and these activities change throughout pregnancy (Tovanabutra et al, 1990). Decidual tissue is likely to be another source of inhibin in pregnancy (Petraglia et al, 1990). Fetal cord blood contains immunoreactive inhibin (McLachlan et al, 1986b), but there is no difference between the levels of inhibin in umbilical artery and umbilical vein and the concentration is only half of the maternal blood value which suggests that the fetus is not contributing to the peripheral immunoreactive inhibin during pregnancy (Kettel et al, 1991; Tabei et al, 1991). Activin A has been detected in amniotic fluid of pregnant women (Abe et al, 1989) and inhibin α-immunoactivity is present in amniotic fluid from 16-20 weeks gestation (Sinosich et al, 1991) which suggests that the feto-placental unit is a source of inhibin. The aims of this study were firstly to examine the immunoactivity of inhibin in amniotic fluid at various stages of pregnancy using three antibodies which recognize different parts of the inhibin molecule, secondly to examine the *in vitro* bioactivity of amniotic fluid inhibin and lastly to verify dimeric inhibin bioactivity by immunoneutralization using an antibody raised against the human inhibin α-subunit.

5.2 Materials & Methods

Amniotic fluids

Amniotic fluids were collected at various stages of gestation: at 7 weeks from pregnancy terminations, 16-17 weeks from amniocentesis and at term from delivery. The amniotic fluid was centrifuged at 1000g, for 15 min at 4°C to remove blood and amniotic fluid cells and was kept at -20°C until assayed.

Radioimmunoassays

Inhibin α-subunit radioimmunoassay using the Y29 antibody, inhibin βA-subunit radioimmunoassay using the R187 antibody and the heterologous inhibin radioimmuno-assay using the Monash antibody were performed as described in Section 2.2 of Chapter 2.

Inhibin *in vitro* bioassay

A sensitive *in vitro* bioassay using sheep pituitary cells was carried out as described by Tsonis et al, 1986 (see Section 4.2 of Chapter 4).

Sample preparation

Amniotic fluids from the same stages of pregnancy were pooled and concentrated to one quarter of their original volume using polyethyleneglycol 6000 (BDH). The concentrated amniotic fluid was charcoal-stripped (10mg/ml amniotic fluid) to remove steroid residues (McLachlan et al, 1986b) and filtered using Millex GS filters (0.22 μm, Waters) prior to the assay.

Assay procedure

As previously described for placental extracts, five doses of amniotic fluid were assayed in quadruplicate ranging from 25-300 μl for 7 weeks or term amniotic fluids and 12.5-100 μl for 16 weeks concentrated amniotic fluid. The inhibin reference standard used was a recombinant human inhibin A with an arbitrary potency of 51 IU/ng (NIH rhINH-R-90/1). A charcoal-Sep-Pak-treated ovine follicular fluid (CGT-1083-oFF) was used as the quality control.

Neutralization of inhibin bioactivity

The *in vitro* neutralization of amniotic fluid was performed using the same basic procedure as outlined in Section 2.2 of Chapter 4. Different doses of charcoal-stripped amniotic fluid from 16 weeks and term were assayed to generate FSH-inhibition curves. Doses producing inhibition ranging from 40-60% were chosen to study the *in vitro* neutralization effect of the Y29 polyclonal antibody. The test materials at the selected doses (400 μl of 16 week amniotic fluid and 133 μl of term amniotic fluid) were incubated with different concentrations of antibody at room temperature for 2 h before being added to the preincubated ovine pituitary cell cultures in triplicate. After making the final incubation volume up to 600 μl with supplemented DMEM, the

culture plates were incubated for a further 48 h and the spent media collected and assayed for FSH.

5.3 Results

Figure 5.1 demonstrates parallelism between the dose-response lines of partially-purified human follicular fluid inhibin standard and amniotic fluids from 7 weeks and 16 weeks of gestation, and delivery, in the heterologous RIA using the Monash antibody against 31 kDa bovine inhibin. The parallelism between the dose-response curves of synthetic human inhibin α-(1-23)-NH_2 and amniotic fluids from 7 weeks, 16 weeks and delivery in the RIA using the Y29 antibody is shown in Figure 5.2. Figure 5.3 also reveals that the dilution curves of synthetic human inhibin β-(97-112)-NH_2 and amniotic fluids from 7 weeks, 16 weeks and delivery are parallel in the RIA using the R187 antibody.

The immunoactivity of inhibin in amniotic fluid was measured by the Monash, R187 and Y29 antibodies. In Figure 5.4, the immunoactivity with the Monash antibody increases towards pregnancy from 135.83 ± 25.10 mU/100 µl, n=9, at 6 weeks to 172.49 ± 20.26 mU/100 µl, n=15, at 16 weeks and 221.49 ± 23.21 mU/100 µl, n=11, at term ($p<0.05$). The immunoactivity with the R187 antibody decreases throughout pregnancy from 450.39 ± 84.36 pg/100 µl, n=9, at 6 weeks to 369.02 ± 30.22 pg/100 µl, n=15, at 16 weeks ($p<0.01$) and 136.84 ± 10.85 pg/100 µl, n=11, at term ($p<0.01$).The immunoactivity with the Y29 antibody also decreases throughout pregnancy from 115.82 ± 17.41 pg/100 µl, n=9, at 6 weeks to 88.49 ± 9.98 pg/100 µl p, n=15, at 16 weeks ($p<0.01$) and 27.52 ± 2.36 pg/100 µl, n=11, at term ($p<0.01$). Values are means ± SEM.

Bioactivity of pooled amniotic fluids (n=5) from 7 weeks, 16 weeks and delivery was measured using the *in vitro* sheep pituitary cell bioassay. The results in Figure 5.5 demonstrate parallelism for the dilution curves of amniotic fluids and human recombinant inhibin A. The bioactivity of inhibin in 7 weeks and term amniotic fluid are 3.86 IU/100 µl and 3.22 IU/100 µl which are higher than in mid-term (0.81 IU/100 µl).

The data from immunoneutralization of bioactive amniotic inhibin are shown in Figure 5.6. The suppressing activity of bioactive inhibin in amniotic fluids from 16 weeks

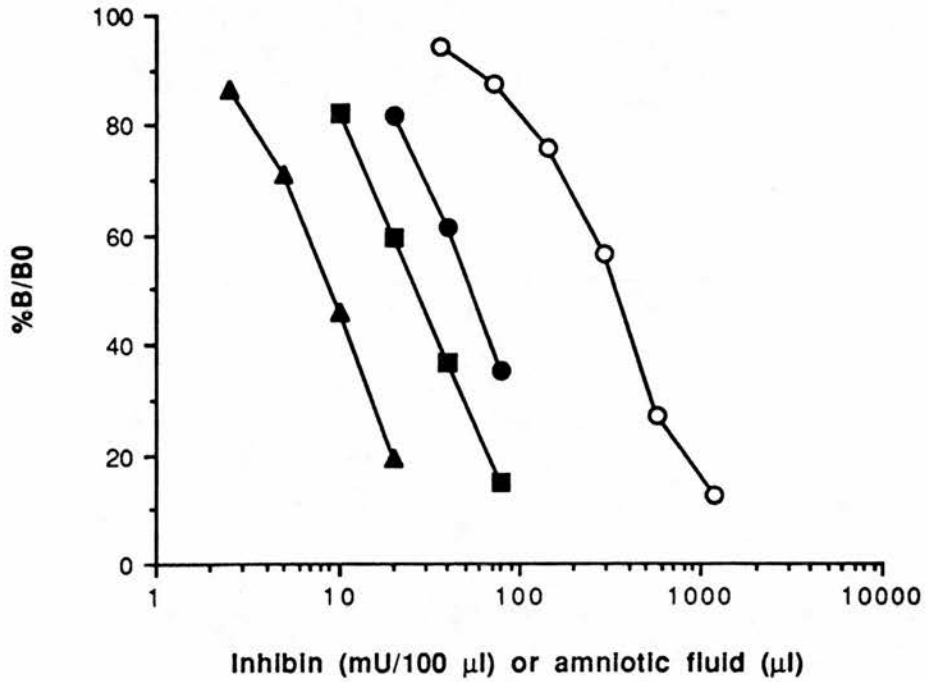

Figure 5.1 Dose-response curves of partially-purified human follicular fluid inhibin standard (O) and amniotic fluids from 7 weeks (●), 16 weeks (■) and delivery (▲) demonstrating parallelism in the heterologous RIA using the Monash antibody.

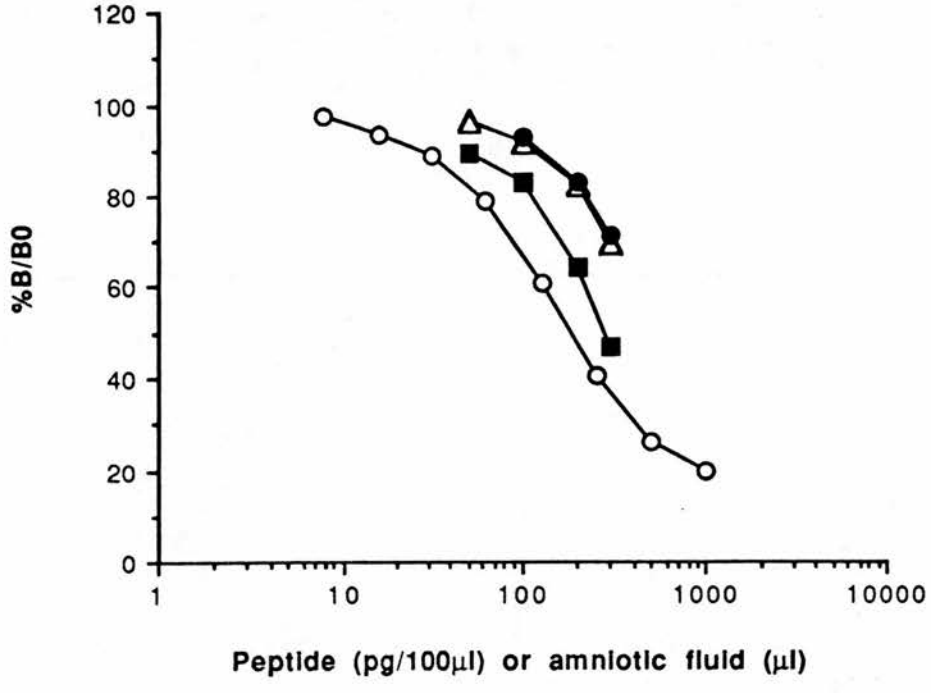

Figure 5.2 Dose-response curves of synthetic human inhibin α-(1-23)-NH_2 (O) and amniotic fluids from 7 weeks (●), 16 weeks (■) and delivery (Δ) demonstrating parallelism in the RIA using the Y29 antibody.

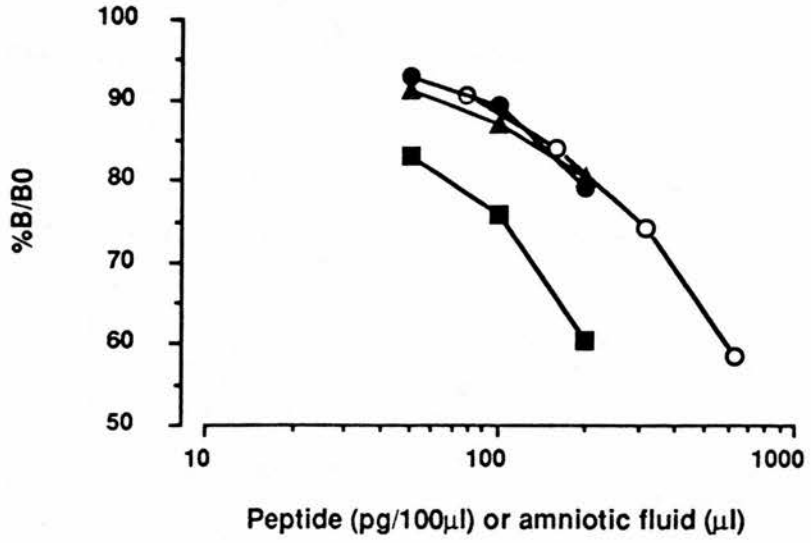

Figure 5.3 Dose-response curves of synthetic human inhibin β-(97-112)-NH_2 (O) and amniotic fluids from 7 weeks (●), 16 weeks (■) and delivery (▲) demonstrating parallelism in the RIA using the R187 antibody.

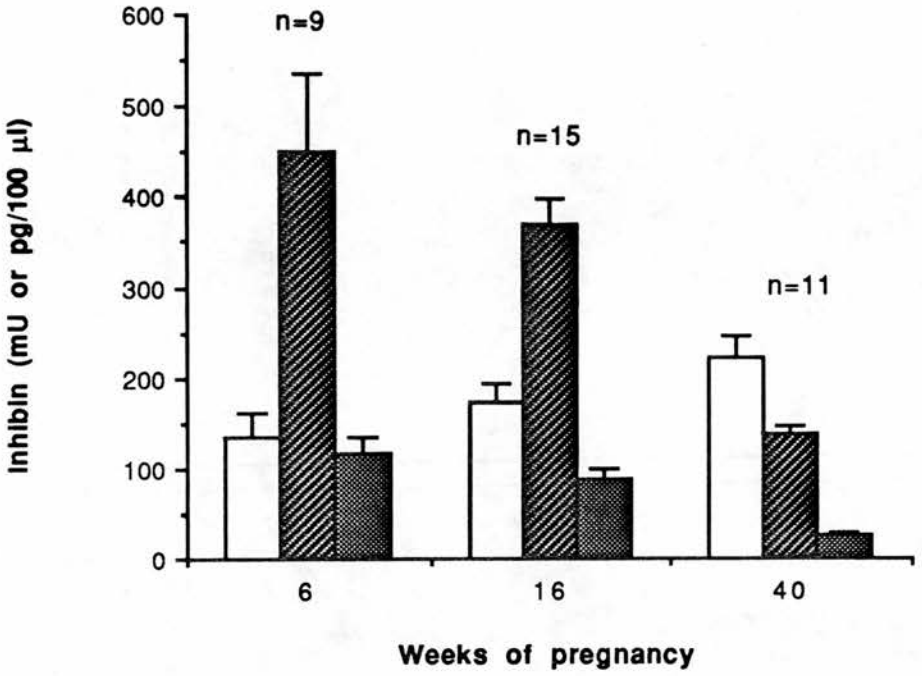

Figure 5.4 Immunoactivity of inhibin in amniotic fluids during pregnancy measured by the Monash antibody (□), R187 (▨) and Y29 (■). Columns represent means ± SEM.

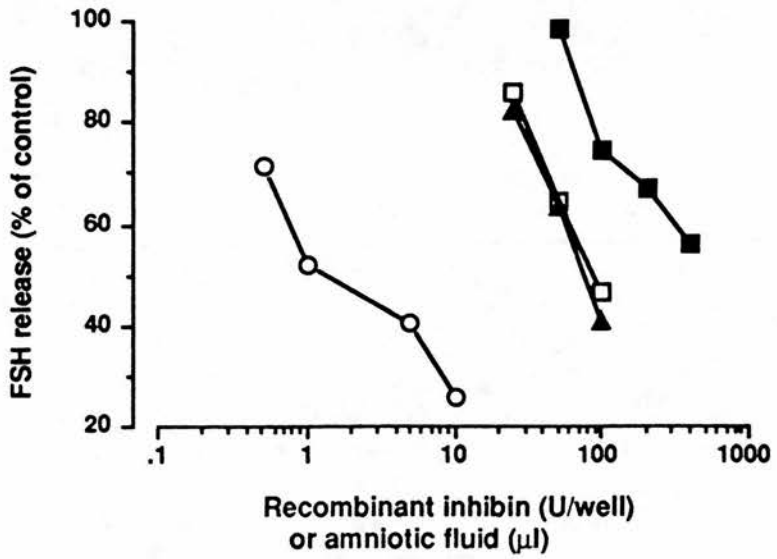

Figure 5.5 Bioactivity of amniotic fluids from 7 weeks (□), 16 weeks (■) and delivery (▲) demonstrating parallelism with human recombinant inhibin A (O).

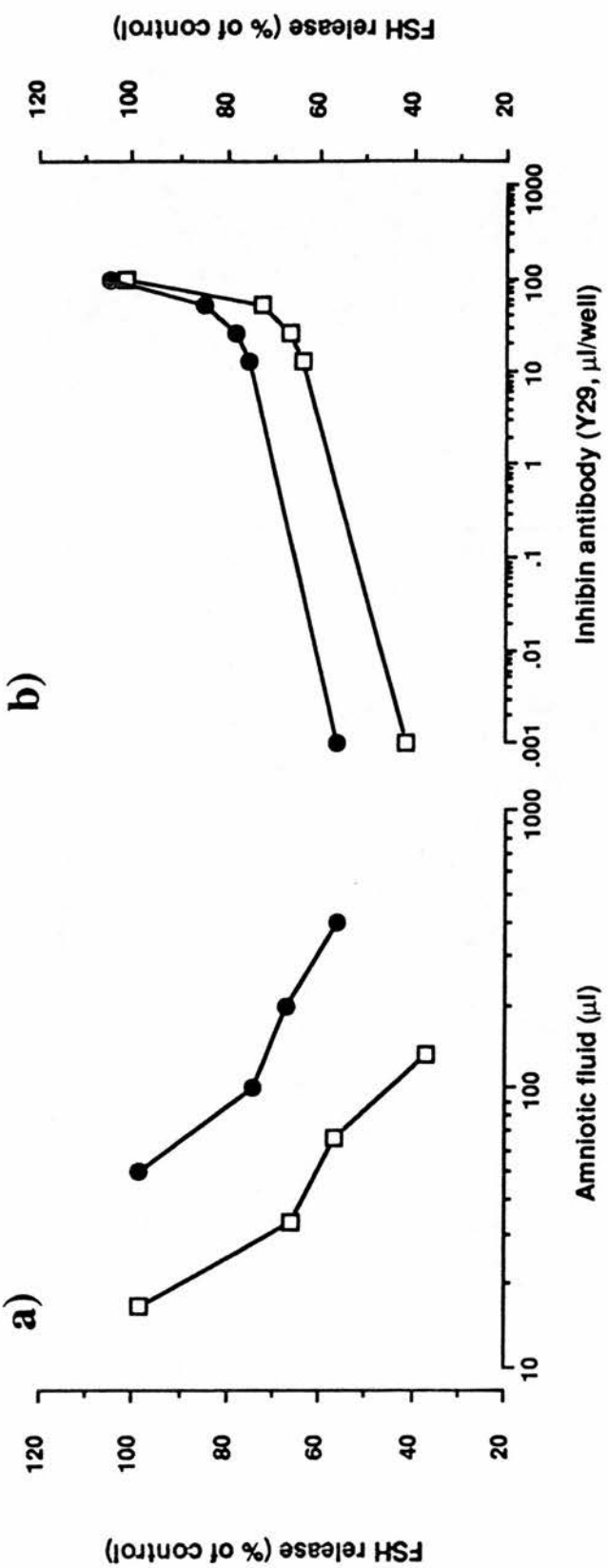

Figure 5.6 (a) The suppressing activity of bioactive inhibin in amniotic fluids from 16 weeks pregnancy (●) and term pregnancy (□) on the FSH secretion of ovine pituitary cells *in vitro*. (b) The reverse effect of inhibin on FSH secretion by immunoneutralization using the Y29 antibody.

pregnancy and term pregnancy on FSH secretion by sheep pituitary cells *in vitro* was reversed by immunoneutralization using the Y29 antibody.

5.4 Discussion

These results demonstrate changes in the immunoactivity of inhibin and its related peptides in amniotic fluid throughout pregnancy. The parallelism of the standard curves and dose-response curves of amniotic fluids from different stages of pregnancy in each RIA show the specificity of the antibodies and the assays.

The profile of inhibin immunoactivity in amniotic fluid measured by RIAs using various antibodies are different. The RIAs with the Y29 and R187 antibodies reveal a decrease in inhibin concentration towards term which is entirely different from the results obtained from the RIA using the Monash antibody which shows that immunoactive inhibin increases towards term.

The level of inhibin bioactivity is different from its immunoactivity level. The lowest bioactivity was observed in mid-pregnancy amniotic fluid whereas similar higher levels were measured in 7 weeks and term amniotic fluids.

At present, it is rather difficult to explain the reasons for the different profiles of bioactivity and immunoactivity of inhibin in amniotic fluids because of its unknown molecular species. Although the antibodies used in the RIAs seem to be specific as shown in the previous section, they all recognize different parts of the inhibin molecule. The Monash and Y29 antibodies recognize the free α-subunit or its protein precursor (pro-αC) as well as dimeric inhibin (Robertson et al, 1989), but the Monash antibody recognizes human follicular fluid inhibin, which is mostly dimeric inhibin (Robertson et al, 1990b), better than the Y29 antibody (see Chapter 2). Thus, it is possible that there are larger amounts of the free α-subunit or pro-αC secreted into amniotic fluid in early pregnancy in comparison to the dimeric inhibin, and when the gestation age increases the dimeric inhibin which is poorly recognized by Y29 is secreted in larger quantities. This hypothesis is in agreement with the results from Chapter 4, suggesting that in the early stages of pregnancy placental tissue produces more free α-subunit or its precursor than in the later stages of pregnancy.

The bioactivity of inhibin pattern is again different from the immunoactivity measured by the Monash antibody. This might due to the presence of non-bioactive inhibin

species as well as bioactive inhibin are produced during pregnancy as mentioned earlier. However, the success in immunoneutralization of inhibin bioactivity using Y29 antibody suggests the presence of real dimeric inhibin in amniotic fluid among other molecular species.

The origin of inhibin in amniotic fluid has not been clearly identified. The human placenta has been demonstrated as a potential source of inhibin (McLachlan et al, 1986b; 1987b; Tovanabutra et al, 1990) and also decidual tissue (Petraglia et al, 1990). Maternal blood and fetal cord blood contain immunoactive inhibin (McLachlan et al, 1986b). These are likely to be possible sources of inhibin in amniotic fluid.

The presence of inhibin activity in amniotic fluid at the very beginning of pregnancy suggests that the fetus may play a role in secreting inhibin at an early stage of life. However, the level of inhibin in umbilical vein and artery are similar and lower than in maternal serum (Kettel et al, 1991; Tabei et al, 1991) suggesting that the fetus is not contributing to the peripheral immunoactive inhibin at least during late pregnancy. Furthermore, there is indirect evidence to support the possible secretion of inhibin by the fetus. The most abundant cells in amniotic fluid are the so-called "typical amniotic cells". These cells arise from fetal membranes and retain some of the properties of the trophoblast such as the synthesis of hCG (Priest et al 1979; 1980). Whether these cells also produce inhibin requires further investigation.

In conclusion, the findings in this chapter provide background information which will be beneficial in the future for further investigations of the role of inhibin in pregnancy.

CHAPTER 6

Immunolocalization of inhibin α- and βA-subunits in human placental tissue at various stages of pregnancy

6.1 Introduction

The presence of inhibin immunoactivity and bioactivity in placenta during pregnancy has been clearly demonstrated in previous chapters of this thesis and by McLachlan and colleagues (McLachlan et al, 1986b). In term placenta, inhibin α-subunit immunoactivity has been localized in cytotrophoblast cells and decidual cells which also contain inhibin βA- and βB- subunits (Petraglia et al, 1990). These findings suggest that during pregnancy, both trophoblast and decidual cells are sources of inhibin or inhibin-related proteins. As described in previous chapters and elsewhere, the concentration of immunoactive inhibin in the human placenta decreases throughout gestation while the level of bioactive inhibin does not change (Tovanabutra et al, 1990).

At present the available radioimmunoassay systems not only measure the intact inhibin molecule but may also measure the free inhibin α-subunit or pro-αC which have no FSH-suppressing bioactivity *in vitro*. Furthermore, the presence of inhibin β-subunit immunoactivity in human placental tissues at various stages of pregnancy has not been clearly demonstrated.

Therefore, the following studies were carried out firstly to obtain methods for inhibin α- and βA-subunit immunolocalization in placenta by modifying the established method for immunolocalization of the inhibin α-subunit in ovarian tissues and the Western immunoblotting method for inhibin β-subunit. Secondly, we hoped to use these methods to investigate the presence and cellular localization of the inhibin βA-subunit in human placenta during pregnancy and to relate this to the localization of the inhibin α-subunit. Moreover, we also wanted to determine whether there are changes in the intensity of inhibin α- and βA-subunit staining in placental tissue which might suggest a differential secretion of these subunits and thus production of intact inhibin, free α-subunit or activin.

6.2 Modification of the inhibin subunit immunostaining methods

6.2.1 Inhibin α-subunit staining

The inhibin α-subunit staining method was established and validated by Smith and co-workers (Smith et al, 1991) to localize the cellular inhibin α-subunit proteins in primate ovaries. This method is an immunoperoxidase technique which uses the highly sensitive avidin-biotin complex as the detection system. The principle of this method is shown in Figure 6.1. Following this method, placental tissue from 7 weeks, 16 weeks and term pregnancy were fixed in 0.1 M sodium cacodylate buffer, pH 7.4 containing 2.5% glutaraldehyde and 4% paraformaldehyde. After conventionally processing to paraffin, the tissues were cut and mounted on to glass slides and then the sections were treated with 3% (v/v) hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity. The sections were washed in 0.05 M Tris-HCl buffer (TBS), pH 7.6 containing 0.9% NaCl and 0.5% (v/v) Triton-X-100 for 10 min, distilled water for 5 min and TBS for 5 min. The sections were incubated with blocking solution containing normal rabbit serum diluted 1:5 in TBS for 30 min to reduce non-specific background staining. The primary antiserum (Y33), a sheep polyclonal antibody raised against synthetic human inhibin α-(1-23)-NH_2, was diluted 1:2000 in TBS and applied to the sections and incubated for 30 min. The sections were washed twice in TBS for 5 min and were then incubated for 30 min with the secondary antibody, biotinylated rabbit anti-sheep immunoglobulin, diluted 1:500 in TBS. After two washes in TBS for 5 min each, peroxidase-conjugated avidin-biotin complex was applied to the tissue sections and incubated for 30 min. The sections were then washed twice in TBS for 5 min. The sections were visualized using 0.5mg 3,3'-diaminobenzidine tetrahydrochloride/ml in TBS containing 0.1ml 3% hydrogen peroxide which was applied to the sections for 3-5 min or until the colour was developed. The sections were then washed in TBS and counterstained in haematoxylin (BDH), dehydrated and mounted using Histomount (National Diagnostics) for DAB treated sections.

The results showed considerably high background staining and very weak intensity staining in trophoblast cells of placental tissues from different stages of pregnancy. The possible factors contributing to low intensity staining were examined as follows:

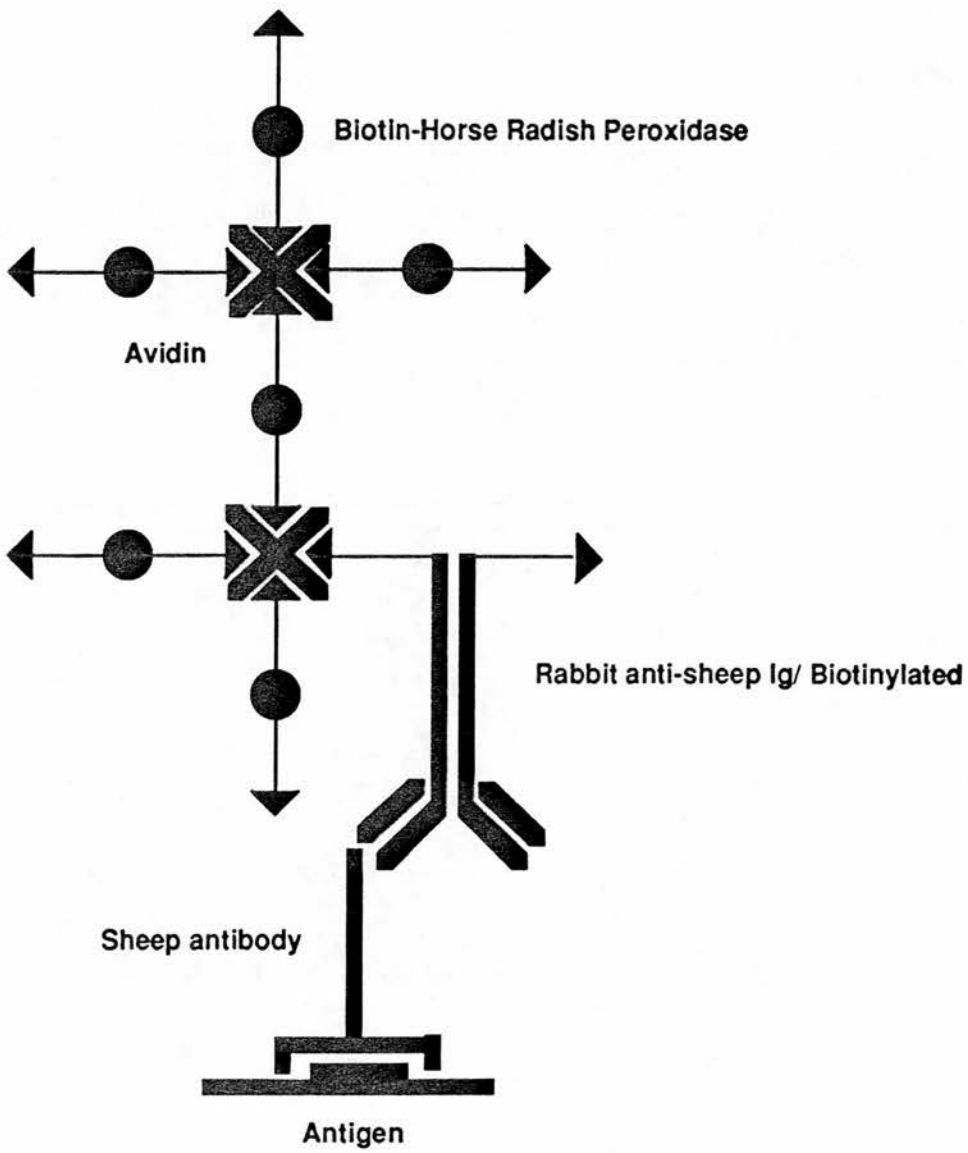

Figure 6.1 Avidin-biotin complex (ABC) method

1. The primary antibody dilution

Apart from the concentration of protein or antigen content in tissue, unsuitable dilution of the primary antibody can cause low intensity staining. To improve the intensity of staining, the antibody was assayed at dilutions of 1:100, 1:250, 1:500 and 1:1000. The results showed that the optimum dilution of antibody was 1:500
which gave a compromise between the intensity of specific staining and acceptable background staining.

2. The fixative

For the optimum fixation of tissues, the ideal fixative should produce good cellular morphology without destroying the immunoactivity of the antigen. The most popular fixative for immunocytochemistry is paraformaldehyde which fixes the tissues by reacting with basic amino acids e.g. lysine and arginine to form cross-linking bridges. This means that there is relatively low permeability to the antibody molecules and their structures are not altered. Glutaraldehyde is another fixative that works in the same way as paraformaldehyde but it reacts more strongly and gives more cross-linkages. The fixative used for the human ovary contains both paraformaldehyde and glutaraldehyde. However, in a loose compact tissue like placenta this fixative may give rise to too many cross-linkages, thus reducing the access of the primary antibody to the antigen molecules. Inhibin α-subunit immunolocalization was previously demonstrated in term placenta using ice-cold 4% paraformaldehyde in phosphate-buffered saline (Petraglia et al, 1987a; Merchenthaler et al, 1987). Under these circumstances, a fixative containing 4% paraformaldehyde in PBS pH 7.4 was used to fix placental tissues and proteolytic digestion using trypsin was introduced into the staining procedure. The final protocol for inhibin α-subunit staining of placental tissue is as shown in Section 6.3.1.3.

6.2.2 Inhibin βA-subunit staining

In combination with the system used for Western immunoblotting analysis of inhibin β-subunit as described in Chapter 2 and the method for localizing the inhibin α-subunit mentioned above, an inhibin βA-subunit staining method was established. The peroxidase-anti-peroxidase complex method was employed as the detection system. The principle of the method is shown in Figure 6.2. To obtain the optimum intensity of staining the antibody was assayed at dilutions of 1:100, 1:200, 1:500, 1:750 and 1:1000. The dilutions which gave strong staining were 1:100 and 1:200, so a dilution of 1:200 was chosen. The final protocol is shown in Section 6.3.1.3.

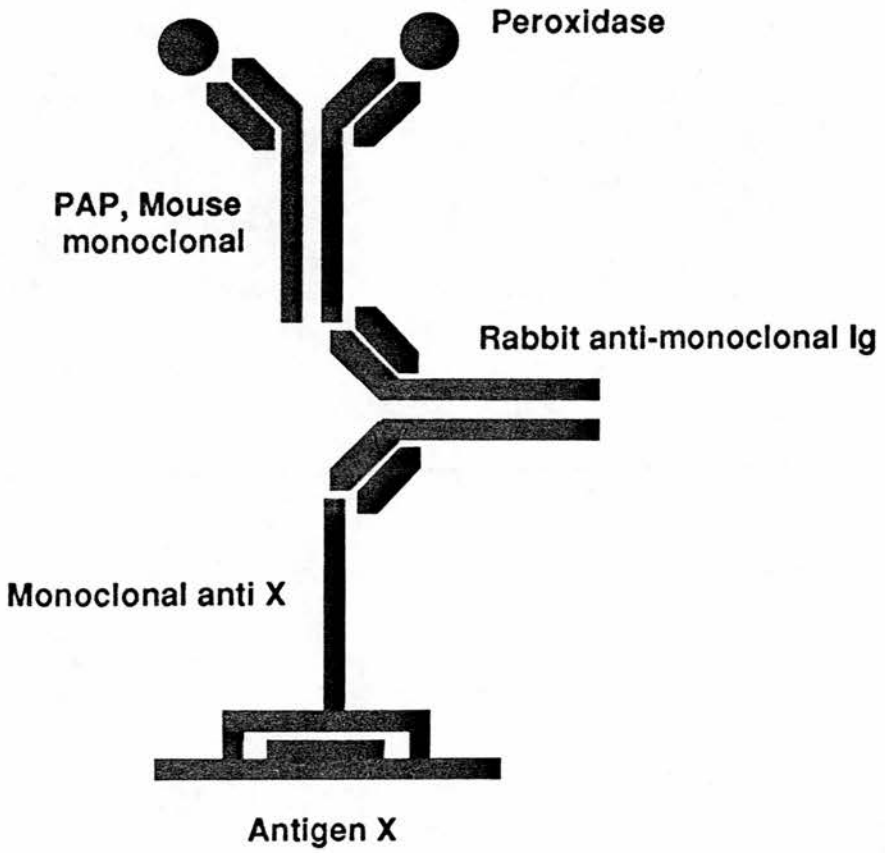

Figure 6.2 Peroxidase-anti-peroxidase (PAP) method .

6.3 Immunolocalization of inhibin α- and βA-subunits in human placental tissue at various stages of pregnancy

The immunocytochemical localization methods obtained above were used to investigate the presence and cellular localization of inhibin subunit proteins in human placental tissue.

6.3.1 Materials and Methods

6.3.1.1 Tissue preparation.

Human placentae were obtained from pregnancy termination at 7 weeks, 16 weeks and at term. Approval of the Local Ethical Committee (Division of Reproductive Medicine, Lothian Health Board) was obtained. The tissues were processed within 30 min of removal. The placentae were cut across the basal plate into slices of approximately 1 cm thickness, and were washed in ice-cold phosphate-buffered saline (PBS, see Appendix 1), pH 7.4 to remove blood. The tissues were fixed in freshly prepared ice-cold 4% paraformaldehyde in PBS, pH 7.4 (see Appendix 1) at 4°C overnight and then dehydrated and embedded in paraffin. Sections of 4 μm thickness were cut, mounted on glass slides and dried at 37°C overnight.

6.3.1.2 Antibodies

Antibody to the inhibin α-subunit

A polyclonal antibody to synthetic human inhibin α-(1-23)-NH_2 (Peninsula Laboratories, San Carlos, CA) was raised in sheep. The synthetic peptide was conjugated to porcine thyroglobulin by the carbodi-imide method (McNeilly et al, 1989). The parallelism between the dose-response curves of a term placental extract, human follicular fluid and the standard curve using human inhibin α-(1-23)-NH_2 in the RIA system is shown in Figure 6.3.

Radioimmunoassay of inhibin in placental extracts from various stages of pregnancy using the Y33 antibody, raised against the amino acids 1-23 N-terminal sequence of synthetic human α-inhibin, and iodinated appropriate peptide as the tracer was performed to check the specific activity of the antibody to inhibin in placental extracts from 7 weeks, 16 weeks and term. To obtain a rapid assay, the RIA system using Y29

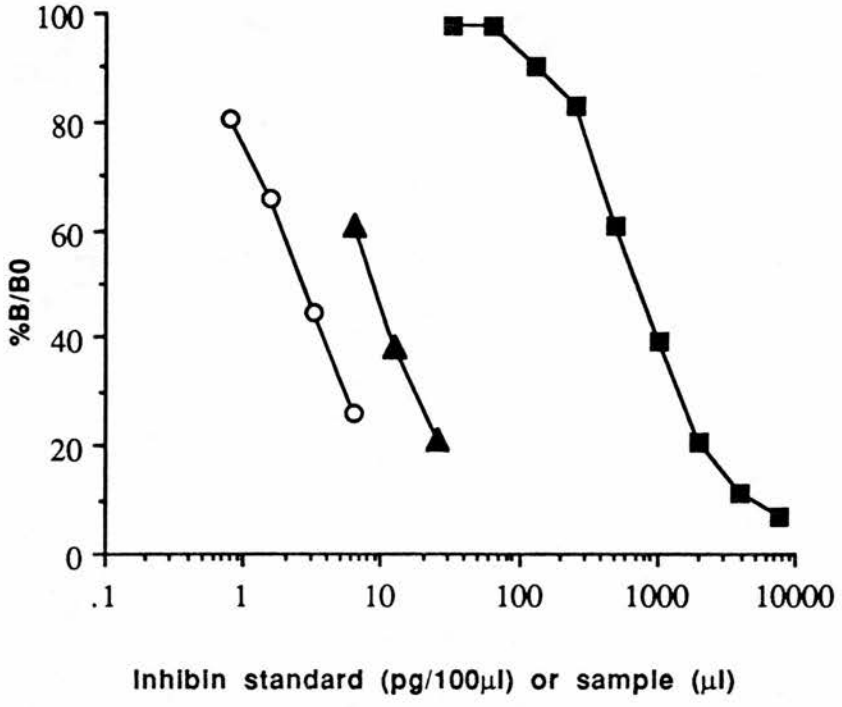

Figure 6.3 Logit-log dose-respose curves of human 1-23 α-inhibin standard (■), term placental extract (O) and human follicular fluid (▲) demonstrating parallelism.

antibody as described earlier in Chapter 2 was modified using primary antibody conjugated to fluorescein thiocyanate (FITC). The antigen-antibody-FITC complexes were separated from free antigen by a second antibody to FITC conjugated to magnetic particles. The antigen-antibody-FITC-anti-FITC magnetic particle complexes were sedimented in a magnetic field. The assay procedure is as follows:

Assay procedure:
The following reagents were added in sequence to the assay tubes:
100 µl of standard (human inhibin 1-23 α-subunit standards, range 31.25-7,800 pg/100 µl) or diluted sample (placental extracts or human follicular fluid).
100 µl of tracer (^{125} I-human 1-23 α-inhibin peptide) containing approximately 15,000 cpm.
100 µl of diluted antibody (1:6000 in assay buffer) conjugated to fluorescein isothiocyanate (FITC), supplied by Serono Laboratories, Woking, Surrey.
Incubation at 37°C for 1 h.
200 µl of anti-FITC magnetic particles (Serono).
Incubation at room temperature for 5 min.
The assay tubes were placed on a magnetic tray for 2 min and the supernantant was discarded by tipping.
The assay tubes including the maximum binding (Bo) and non-specific binding (NSB) tubes were counted and the results were calculated in the same way as the other radioimunoassays described in Chapter 2.

Antibody to inhibin βA-subunit
A monoclonal antibody against amino acids 84-112 of the human synthetic βA-inhibin subunit was raised in mouse. The peptide was coupled to tuberculin via the -SH group using protocols obtained in a kit available from Cambridge Research Biochemicals, Button End Industrial Estate, Harston, Cambridge, U.K (Groome & Lawrence, 1991). This antibody has been shown to localize the inhibin β-subunit in human corpus luteum (Smith et al, 1992)

6.3.1.3 Immunocytochemistry

Paraffin sections were deparaffinised in xylene and rehydrated in graded ethanol. The tissue sections were incubated with 3% H_2O_2 (BDH) in methanol (BDH) for 30 min to block any endogenous peroxidase activity. The sections were washed with two changes of 0.05 M Tris-HCl buffer, pH 7.6 (TBS). The sections were incubated for 15 min at

37°C in 0.1% trypsin (BDH) and 0.1% $CaCl_2$ (BDH) in water and then washed in 0.5 % Triton X-100 (BDH) in TBS for 10 minutes. Following two 5 min washes in TBS, the sections were incubated for 30 min with the blocking solution [normal rabbit serum (Dako Ltd.), diluted 1:5 in TBS containing 5% BSA] to minimize non-specific background staining.

Inhibin α–subunit staining

The sections were incubated with the primary antibody diluted 1:500 in blocking solution for 30 min at room temperature. After washing with TBS twice (5 min each), the sections were incubated for 30 min with second antibody, biotinylated rabbit anti-sheep immunoglobulin (Vector Laboratories, Bretton, Peterborough) diluted 1:500 in blocking solution. The sections were washed twice in TBS for 5 min and then incubated with the avidin-biotin peroxidase complex (DAKOPATTS, Dako Ltd.) for 30 min. The sections were washed again with TBS and the colour reaction developed by incubating the sections with 0.05M Tris buffer (pH 7.6) containing 0.05% 3, 3' diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H_2O_2 (BDH) for 3-5 min. The sections were washed with water and then counterstained with haematoxylin (BDH). Finally, the sections were mounted using Histomount (National Diagnostics) and microscopically examined. Specific binding was evaluated by replacing the primary antibody with 1) normal sheep serum (Vector Laboratories) at the same dilution of primary antibody and 2) the appropriate synthetic peptide-preadsorbed antibody.

Inhibin β-subunit staining

The sections were incubated with the primary antibody diluted 1:200 in blocking solution at 4°C overnight. After washing twice with TBS (5 min each), the sections were incubated with rabbit anti-mouse immunoglobulin (Dako Ltd.) diluted 1:50 in blocking solution for 30 min. The sections were again washed with TBS twice and incubated with peroxidase-monoclonal mouse-peroxidase complexes (Dako Ltd.) diluted 1:100 in blocking solution for 30 min. The sections were again washed twice with TBS and the colour developed as described for α-subunit staining. To check the specificity of binding, the preadsorbed βA-subunit antibody with 200 μg/ml of synthetic human βA-(82-114) was used in place of the primary antibody. The intensity of staining was assessed independently by four persons working on immunohisto-chemistry.

6.3.2 Results

As shown in Figures 6.4, 6.6 and 6.8, the trophoblast cells of placental tissues from 7 weeks, 16 weeks and term were positively stained for the inhibin α-subunit. The specificity of the staining was confirmed by the absence of staining with non-immune serum. In Figures 6.5 and 6.7 both syncytiotrophoblast and cytotrophoblast cells were clearly demonstrated in 7 weeks and 16 weeks placentae under high magnification. In term placenta (Figure 6.9) where it is difficult to distinguish between syncytiotrophoblast and cytotrophoblast cells, the trophoblast shell showed positive staining.

Figures 6.10, 6.11, 6.12, 6.13, 6.14 and 6.15 show positive staining of inhibin βA-subunit in placentae during pregnancy. Inhibin βA-subunit staining in 7 weeks, 16 weeks and term placental tissues was demonstrated within the same cell types as inhibin α-subunit staining. Specific staining was absent when the primary antibody was replaced by the antibody preadsorbed with the appropriate peptide.

Some mesenchymal cells in 16 weeks and term placental tissues showed faint positive staining with both antibodies. Furthermore, in mid- and term placentae the endothelial cells lying in fetal blood vessels also showed positive staining with the inhibin α- and βA-subunit antibodies. The pattern of staining is shown in Figure 6.17. The extravillous cytotrophoblast in term placenta also showed positive staining with inhibin α- and βA-subunit antibodies. The staining with βA-antibody is shown in Figure 6.16. Although positive staining for both inhibin α- and βA-subunits was shown in trophoblast cells throughout pregnancy, positive staining for these two subunits varied with the stage of pregnancy, as determined by an arbitrary scoring system (Table 6.1). Maximum staining of inhibin α-antibody was shown in early pregnancy while the intensity of inhibin βA-subunit staining increased towards term.

6.3.3 Discussion

This study demonstrates the presence of both inhibin α- and βA-subunit immunoactivity in trophoblast cells throughout pregnancy. Although inhibin α-subunit immunoactivity has been localized in human cytotrophoblast cells (Petraglia et al, 1987a; Merchenthaler et al, 1987) our results demonstrate that the inhibin α-subunit is also localized in syncytiotrophoblast cells. Furthermore, our results indicate for the first

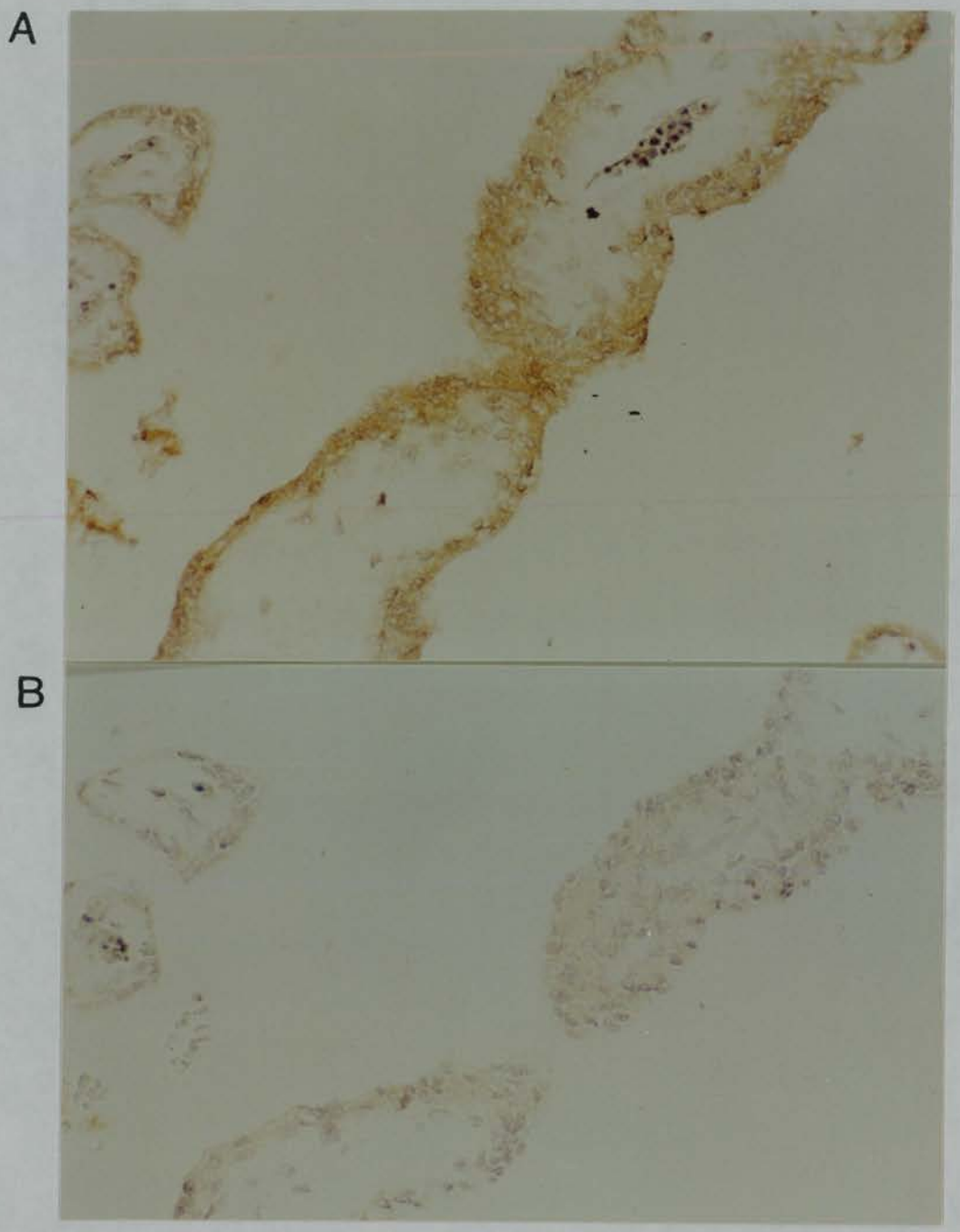

Figure 6.4 Immunolocalization of inhibin α-subunit in 7 weeks placental tissue. (A) Low-magnification photomicrograph (x20) showing positive staining in trophoblast cells with primary antibody and (B) no specific staining in control section with non-immune sheep serum.

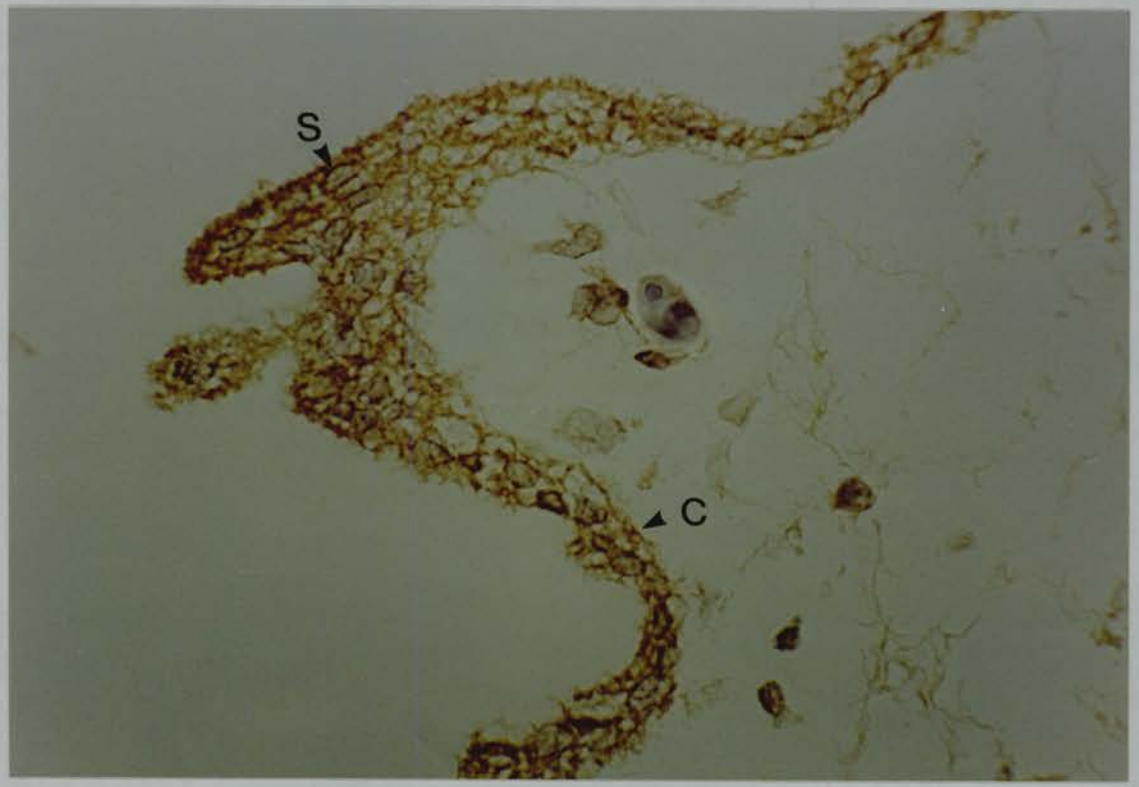

Figure 6.5 High-magnification photomicrograph (x40) showing the localization of inhibin α-subunit in cytotrophoblast (C) and syncytiotrophoblast (S) cells of 7 weeks placental tissue.

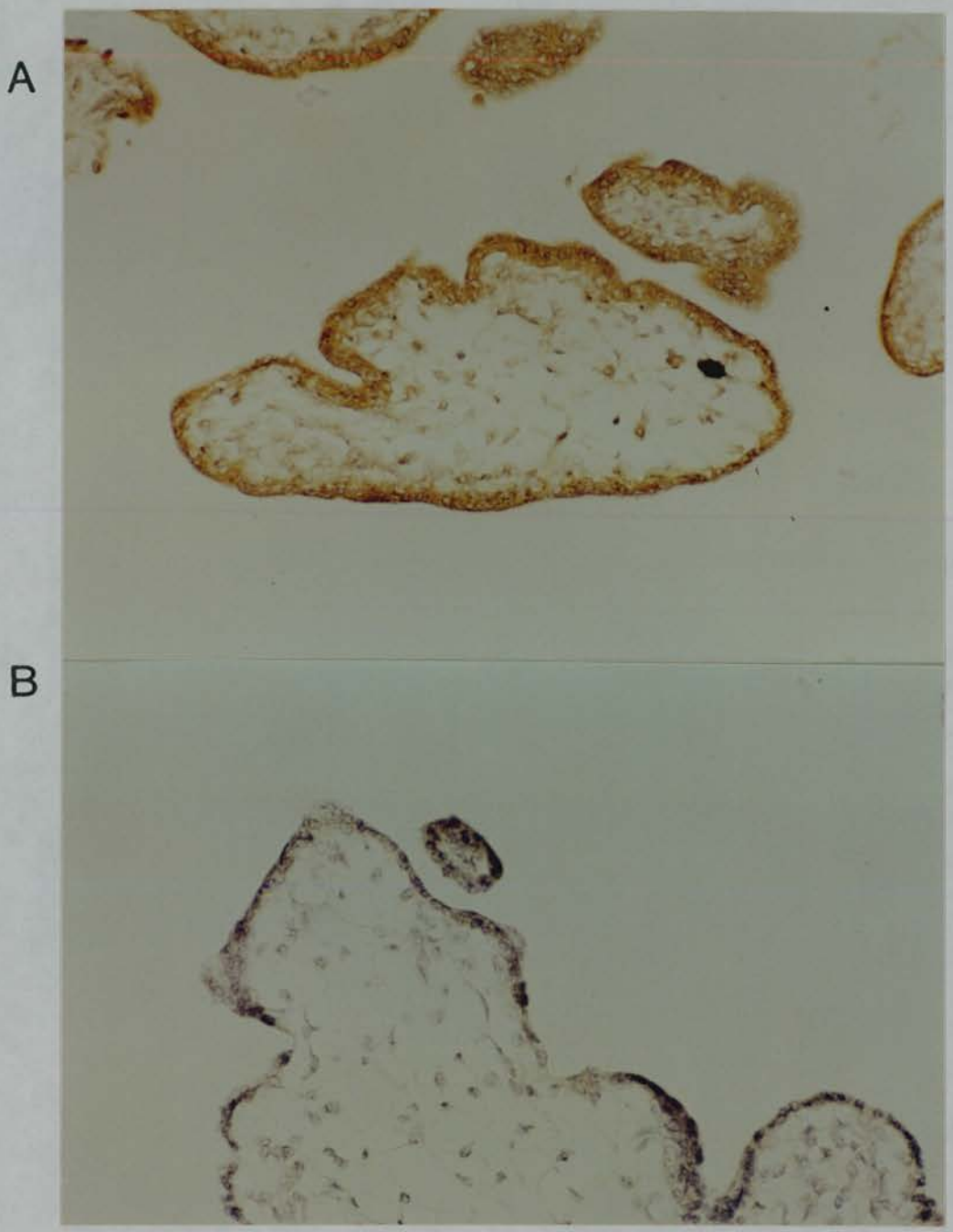

Figure 6.6 Immunolocalization of inhibin α-subunit in 16 weeks placental tissue. (A) Low-magnification photomicrograph (x20) showing positive staining in trophoblast cells with primary antibody and (B) no specific staining in control section with non-immune sheep serum.

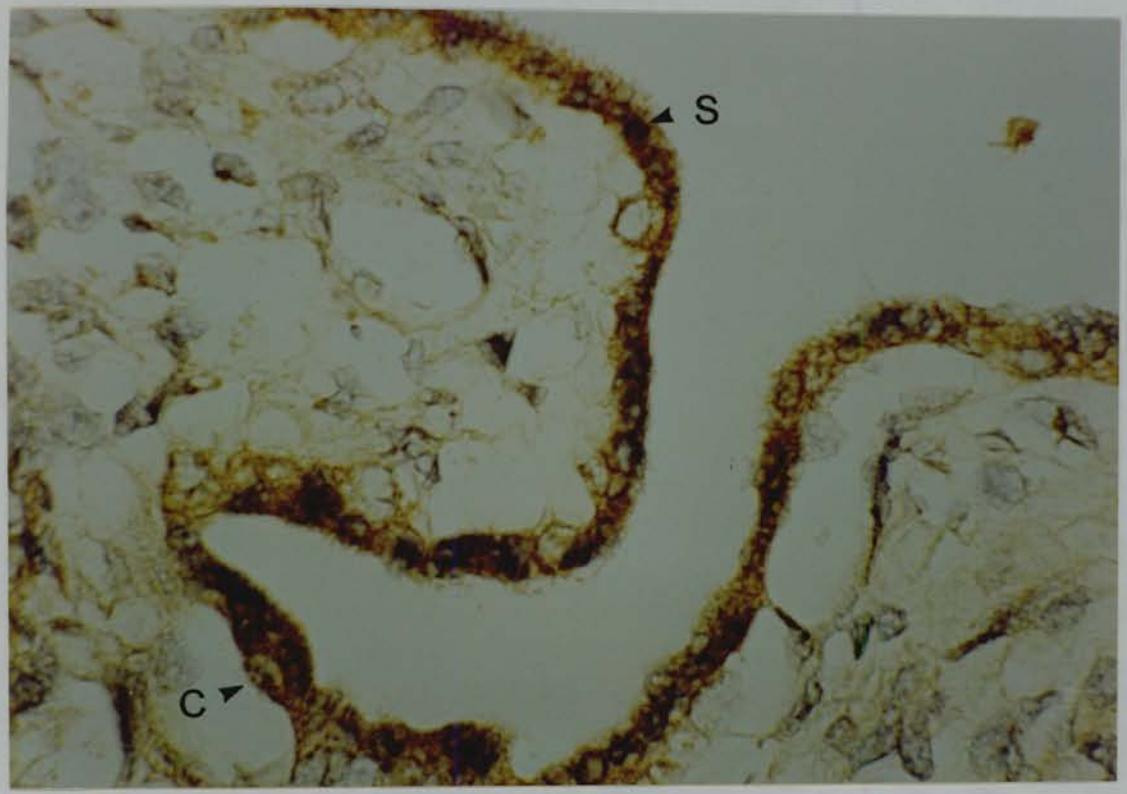

Figure 6.7 High-magnification photomicrograph (x40) showing the localization of inhibin α-subunit in cytotrophoblast (C) and syncytiotrophoblast (S) cells of 16 weeks placental tissue.

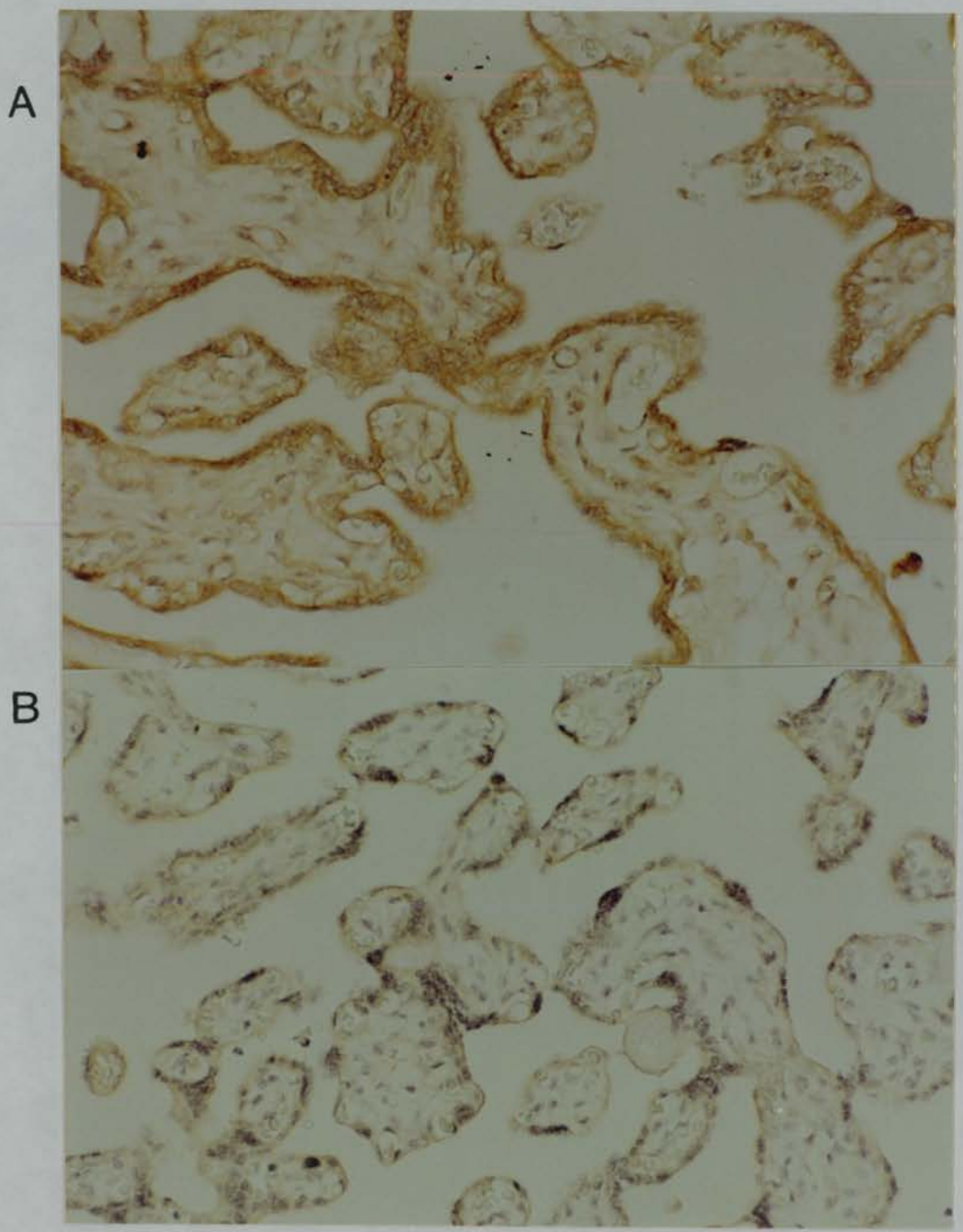

Figure 6.8 Immunolocalization of inhibin α-subunit in term placental tissue. (A) Low-magnification photomicrograph (x20) showing positive staining in trophoblast cells with primary antibody and (B) no specific staining in control section with non-immune sheep serum.

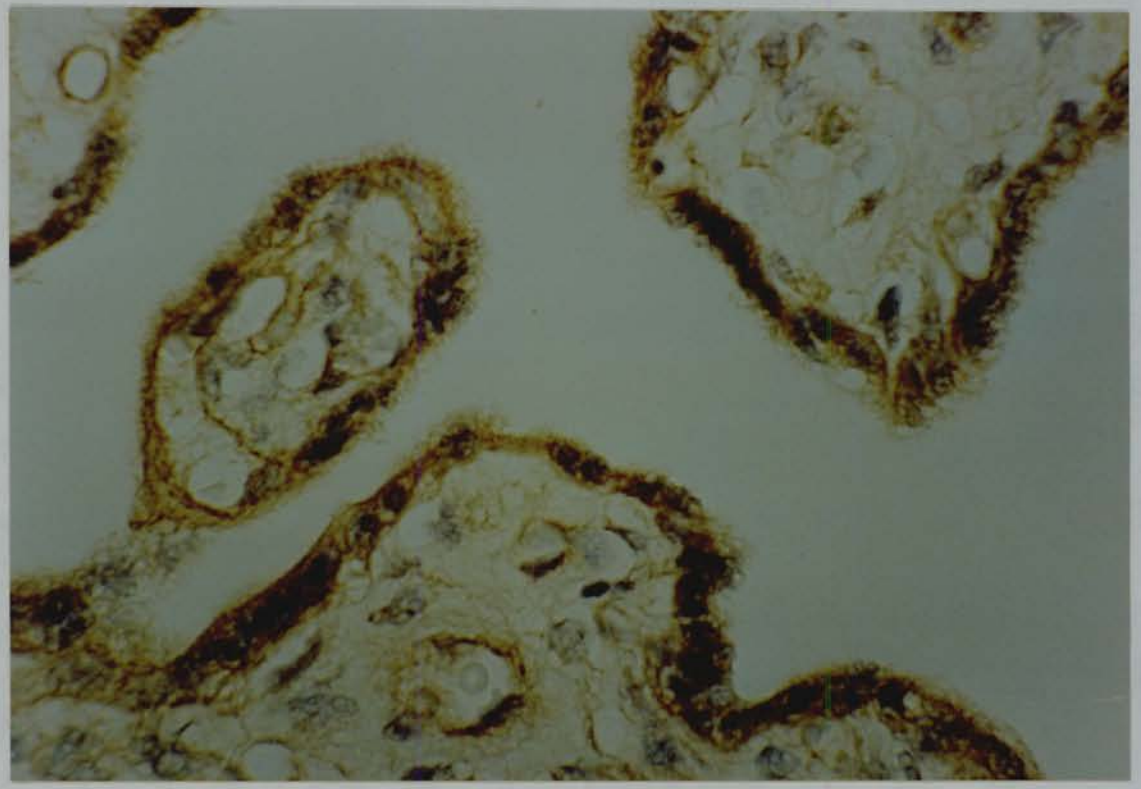

Figure 6.9 High-magnification photomicrograph (x40) showing the localization of inhibin α-subunit in trophoblast cells of term placental tissue.

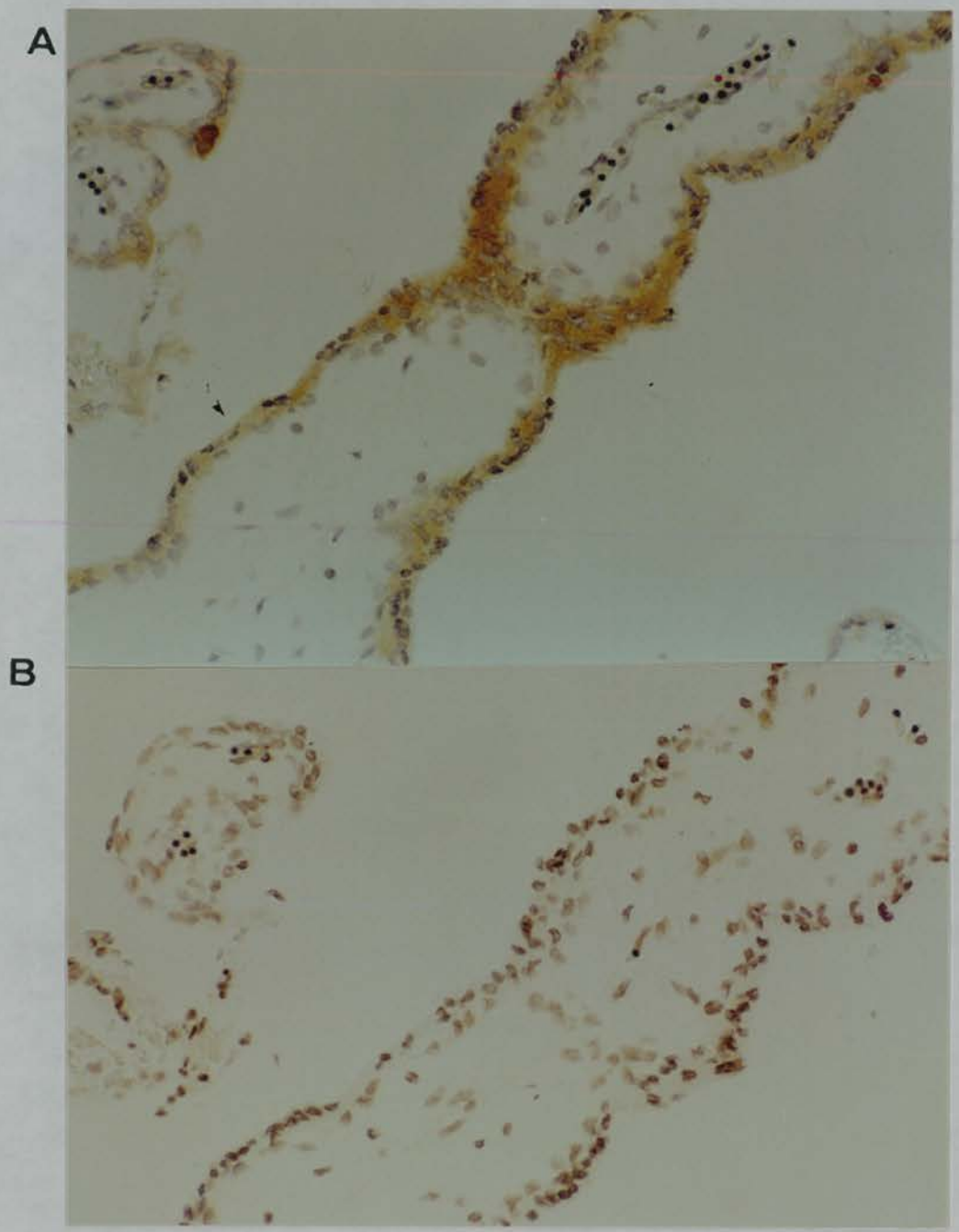

Figure 6.10 Immunolocalization of inhibin βA-subunit in 7 weeks placental tissue. (A) Low-magnification photomicrograph (x20) showing positive staining in trophoblast cells with primary antibody and (B) no specific staining in control section with the appropriate synthetic peptide-preadsorbed antibody.

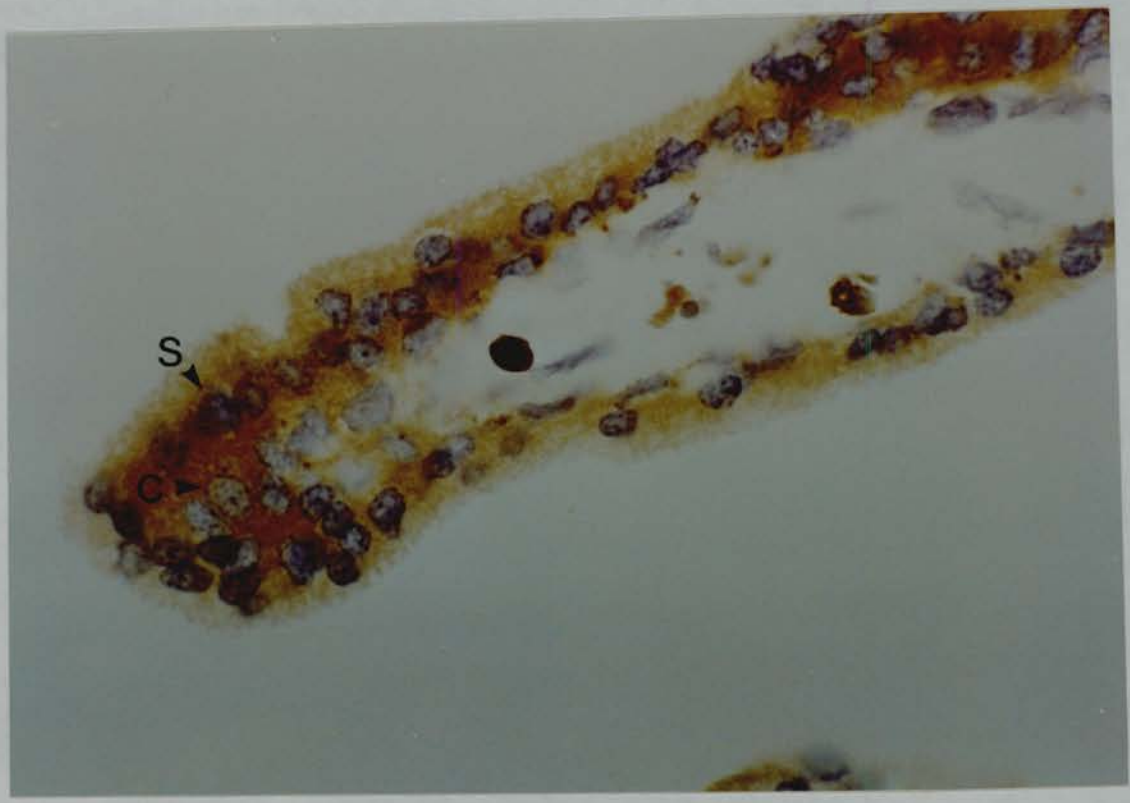

Figure 6.11 High-magnification photomicrograph (x40) showing the localization of inhibin βA-subunit in cytotrophoblast (C) and syncytiotrophoblast (S) cells of 7 weeks placental tissue.

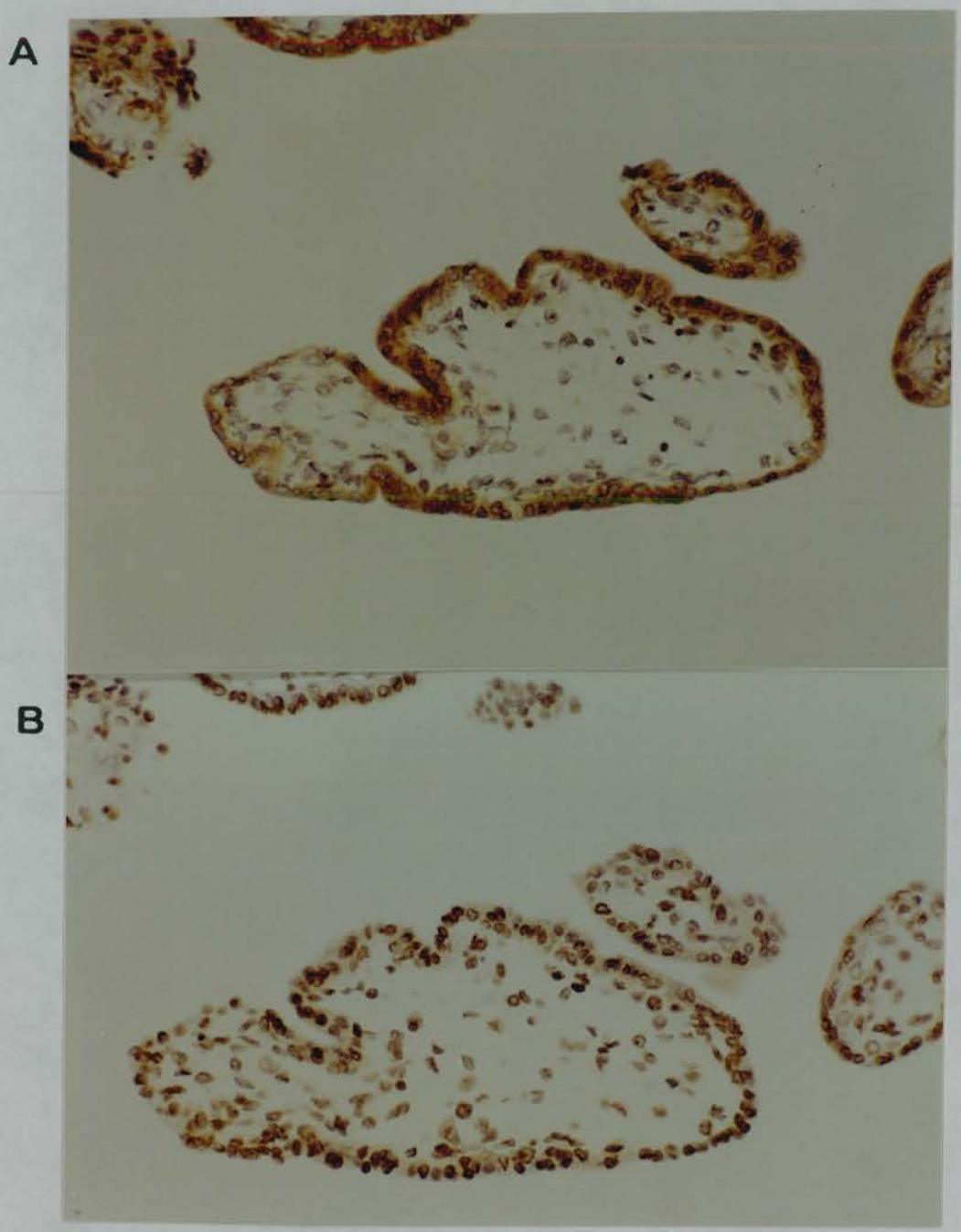

Figure 6.12 Immunolocalization of inhibin βA-subunit in 16 weeks placental tissue. (A) Low-magnification photomicrograph (x20) showing positive staining in trophoblast cells with primary antibody and (B) no specific staining in control section with the appropriate synthetic peptide-preadsorbed antibody.

Figure 6.13 High-magnification photomicrograph (x40) showing the localization of inhibin βA-subunit in cytotrophoblast (C) and syncytiotrophoblast (S) cells of 16 weeks placental tissue.

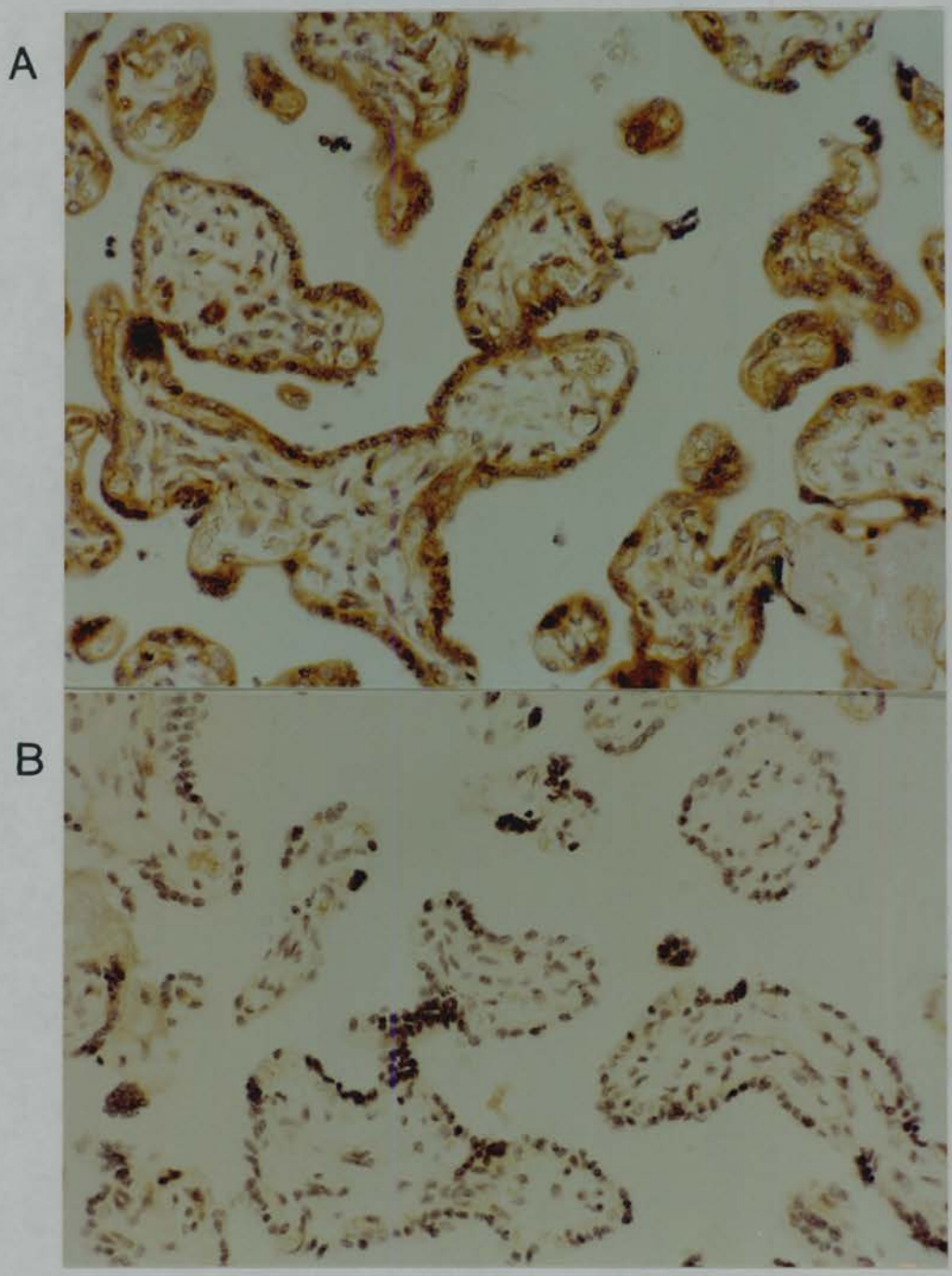

Figure 6.14 Immunolocalization of inhibin βA-subunit in term placental tissue. (A) Low-magnification photomicrograph (x20) showing positive staining in trophoblast cells with primary antibody and (B) no specific staining in control section with the appropriate synthetic peptide-preadsorbed antibody.

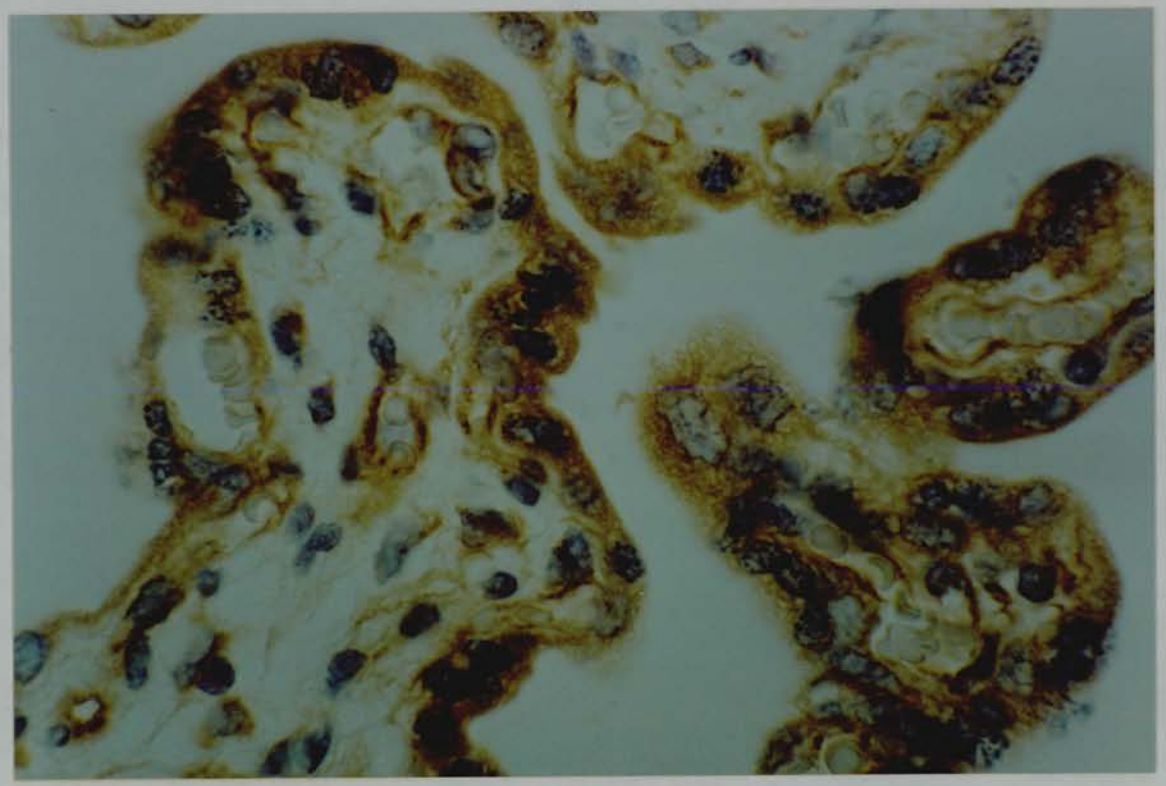

Figure 6.15 High-magnification photomicrograph (x40) showing the localization of inhibin βA-subunit in trophoblast cells of term placental tissue.

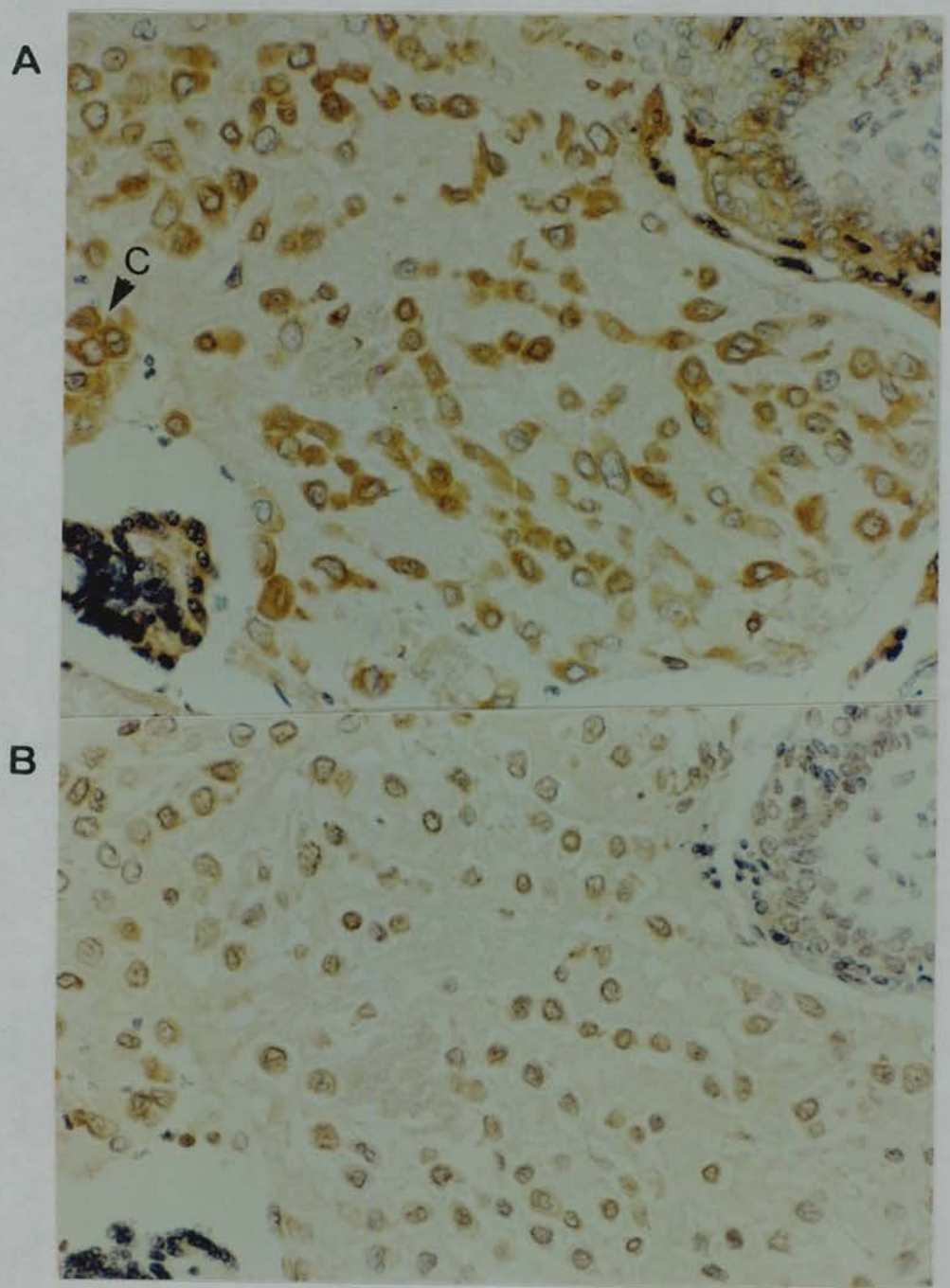

Figure 6.16 Localization of inhibin βA-subunit in term placenta. (A) Extravillous cytotrophoblast cells (C) show intense staining with monoclonal antibody and (B) no staining with the preadsorbed antibody. Magnification x20.

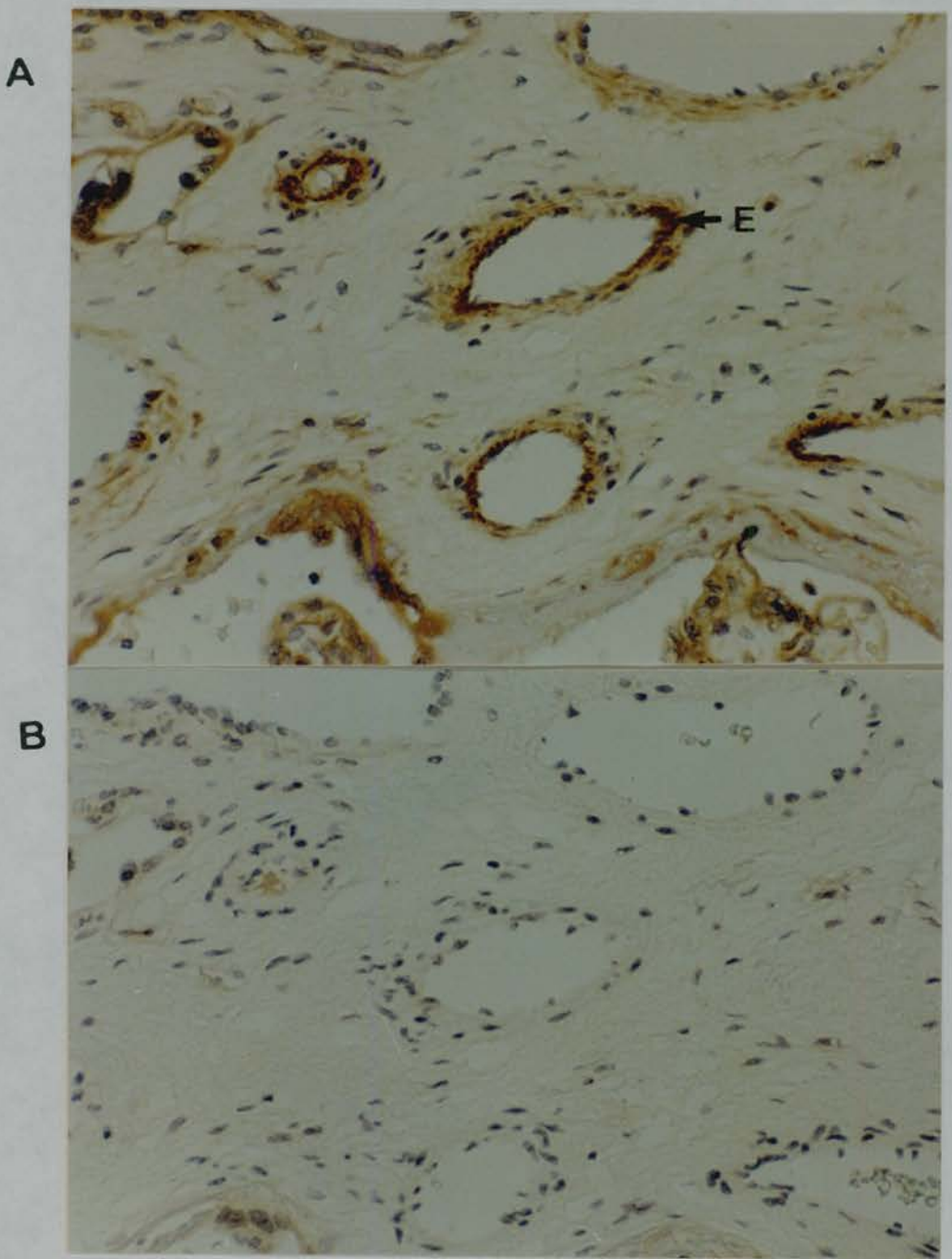

Figure 6.17 Immunolocalization of inhibin βA-subunit in term placental tissue. The endothelial cells (E) lying in fetal blood vessels show very specific intense staining (A) and the staining is not present in the control section (B). Magnification x20.

Table 6.1 The relative positive staining intensities of trophoblast cells in placentae from various stages of pregnancy to inhibin α- and βA- subunit antibodies.

Inhibin subunit	**Age of pregnancy**		
	7 weeks	**16 weeks**	**Full term**
α	+++	+	++
βA	+	++	+++

The intensities of staining were graded ;
+ positive staining, ++ strong staining and +++ very strong staining.

time that inhibin βA-subunit is present in both cytotrophoblast and syncytiotrophoblast cells and this result is supported by the expression of inhibin βA-subunit mRNA in human term placenta (Davis et al, 1987). The existence of both inhibin α- and βA-subunits in the same cell type provides evidence that the placenta produces biologically active intact inhibin as well as the free α-subunit.

The fact that the intensity of staining to inhibin βA-subunit antibody in trophoblast cells increases during pregnancy suggests that the trophoblast cells produce more inhibin βA-subunit as the gestational age increases. Therefore, these differences in the degree of staining of both inhibin subunits in placental tissues from various stages of pregnancy suggest that the human placenta may produce different amounts of various inhibin-like molecules during pregnancy. The strong staining of inhibin α-subunit and reduced staining of inhibin βA-subunit in early pregnancy may indicate a greater production of free α-subunit or pro-αC than intact inhibin at this stage. Meanwhile, the lower staining of inhibin α-subunit and stronger staining of βA-subunit in mid- and term placentae suggest that activin may be the major inhibin-related protein in the later stages of pregnancy. This hypothesis is supported by an earlier finding that inhibin bioactivity in placental extracts does not change during pregnancy while both inhibin and free α-subunit immunoactivity decrease (Tovanabutra et al, 1990).

At present, the physiological role of inhibin and inhibin-related peptides in pregnancy remains unclear. Our results suggest the concept of an autocrine/paracrine role for inhibin and/or activin within the trophoblast. This concept is consistent with current evidence that inhibin has a paracrine role in the regulation of placental hCG production by syncytiotrophoblast cells (Petraglia et al, 1987a). Furthermore, activin enhances the stimulatory action of GnRH on hCG secretion in human placental cell cultures and this effect is reversed by inhibin (Petraglia et al, 1989). The presence of both inhibin subunits in the endothelial cells of fetal blood vessels in mid-term and term pregnancy suggests a possible role of inhibin or activin in fetus development. This is supported by the finding that human recombinant activin A inhibits proliferation of human fetal adrenal cells *in vitro* and inhibin α-, βA- and βB-subunits have been localized in human fetal adrenal glands (Spencer et al, 1990). However, the localization of inhibin α- and βA-subunits in the same cell type and the change in their proportions throughout pregnancy further suggests that the relative roles or requirements for inhibin and activin may change during pregnancy.

CHAPTER 7

Inhibin α- and β-subunit mRNA expression in placental tissue from various stages of pregnancy

7.1 Introduction

Northern blot analysis is a useful approach to demonstrate gene expression in many experimental models. In the last decade, many studies have employed this technique to demonstrate inhibin α-, βA- and βB- subunit mRNA expression in various cells/tissues from different species. In the human, inhibin α-subunit cDNA has been isolated from a placental cDNA library (Mayo et al, 1986). Davis and colleagues (1987) have shown the expression of inhibin α- and βA- but not βB-subunit mRNAs in term placenta. However, Reddi and colleagues (1990b) have been able to demonstrate α- and βB-subunit gene expression in term placenta by Northern blot analysis. Petraglia et al (1990) have also shown that inhibin α-, βA- and βB-subunit mRNAs are expressed in decidual tissue throughout pregnancy by Northern blot analysis. These findings taken together with the results presented in previous chapters of this thesis suggest that secretion and synthesis of inhibin subunits occurs within the feto-placental unit.

The aims of the current study were firstly to investigate inhibin gene subunit expression in the human placenta throughout pregnancy and secondly to use Northern blot analysis to examine the expression of mRNAs for the different inhibin subunits in placental tissue at various stages of pregnancy.

7.2 Expression of inhibin α- and βB-subunit genes in placenta during pregnancy

7.2.1 Materials and Methods

Tissue preparation

Human placental tissue was obtained from pregnancy termination at 7 weeks, 16 weeks and term. The tissue was collected in ice-cold PBS and transported at once to the laboratory. Tissue was cut and weighed, then immediately processed for RNA

extraction or quickly frozen and kept in liquid nitrogen until the RNA extraction was carried out.

Northern blot analysis

RNA extraction

RNA extraction was performed using acid guanidinium thiocyanate-phenol-chloroform as described by Chomczynski & Sacchi (1987). The preparation was kept on ice at all times. All glassware, plasticware and reagents were RNAse-free. 1 g of fresh or frozen tissue was completely homogenized in 10 ml of ice-cold denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol (see Appendix 2.1) using a Polytron homogenizer at speed 8. The homogenate was transferred to sterile polypropylene tubes (Falcon 2059) and 0.1 ml of 2 M sodium acetate, pH 4.0, 10 ml of water-saturated phenol (see Appendix 2.2) and 0.2 ml of chloroform (Sigma)/isoamyl alcohol (Sigma) mixed 49:1 were added to each 10 ml of homogenate. The solution was mixed throughly by inverting after each addition and finally mixed vigorously for at least 10 sec before being placed on ice for 15 min. The solution was centrifuged at 10,000 g for 20 min at 4°C, then the RNA-containing aqueous phase was transferred to a new polypropylene tube and 10 ml cold isopropanol (Sigma) added. The solution was kept at -20°C for 1h to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 g for 20 min at 4°C. After discarding the supernatant, the RNA was redissolved in 0.3-1 ml of denaturing solution by vortexing. The RNA solution was transferred to a 1.5 ml microcentrifuge tube and mixed with an equal volume of cold isopropanol. The RNA was reprecipitated at -20°C for 1h and collected by centrifugation at 13,000 g for 10 min at 4°C. The supernatant was discarded, and the RNA washed with 1 ml 75% EtOH (Aristar, BDH) then centrifuged at 13,000 g for 2 min before careful removal of the supernatant. The RNA pellet was air-dried and dissolved in 50-100 µl of sterile RNase-free water by warming at 65°C for 10 min. The preparation was stored at -70°C until electrophoresis was performed.

Preparation of poly (A)$^+$ RNA

Poly (A)$^+$ RNA was prepared from total RNA by affinity chromatography on an oligo (dT)-cellulose matrix. 80 µg oligo (dT) cellulose P-L type 7 (Pharmacia) was reconstituted in loading buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 1mM EDTA and

0.1% SDS) and packed in a sterile minicolumn (Pharmacia). The column was washed with 3 bed volumes of 0.1M NaOH with 5mM EDTA and then with at least 10 volumes of sterile water until the pH of the effluent was less than 8.0. The column was equilibrated with loading buffer. Approximately 1 mg total RNA was dissolved in 500 µl of sterile water by heating at 65°C for 5 min, cooled to room temperature for 2 min and mixed with 500 µl 2x loading buffer. The RNA solution was applied to the column and washed through with 1 ml of loading buffer and the effluent collected. The RNA-containing effluent was reheated at 65°C for 5 min and reapplied to the column. The column was washed with 5 ml of washing buffer containing 10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1mM EDTA and 0.1% SDS. Poly $(A)^+$ RNA was eluted from the matrix with 1.5 ml of elution buffer (10 mM Tris-HCl pH 7.5, 1mM EDTA, 0.05 % SDS) and collected as four fractions. The mRNA in each fraction was mixed with 1/10th volume of 3 M sodium acetate (pH 5.5) and precipitated with 2.5 volumes of cold ethanol at -70°C for 2 h. The RNA was pelleted by centrifugation at 13,000 g for 10 min at 4°C, air-dried, redissolved in 20 µl sterile water and stored at -70°C until electrophoresis was carried out.

Electrophoresis of RNA on denaturing formaldehyde/agarose gels

4.4 µl of RNA sample containing 20 µg of total RNA or 5 µg of poly $(A)^+$ RNA (see Appendix 2.3) was added to 15.6 µl of loading buffer (see Appendices 2.4 and 2.5) and then denatured at 65°C for 5 min. Prior to loading on to a 1.5% formaldehyde agarose gel (see Appendices 2.6 and 2.7), 8 µl of dye solution containing 7.5% Ficoll 400 (Sigma), and 0.1% bromophenol blue (Sigma) and 1 µl of 1mg/ml ethidium bromide (Sigma) were added. The gel was electrophoresed at 50 V overnight in running buffer. The gel was visualized and photographed under a UV-transilluminator with a ruler to locate the position of ribosomal 28S (5kb) and 18S (2kb) species.

Transfer of RNA to nitrocellulose filter

The electrophoresed RNA was transferred immediately from the agarose gel on to a Hybond-N filter (Amersham) by capillary elution as described by Thomas (1980). The blotting apparatus was set up as described by Southern (1975). Briefly, the gel was placed on a Whatman 3MM paper wick (Figure 7.1). A Hybond-N membrane (Amersham) was prewetted in sterile water and placed over the gel carefully so as to avoid air bubbles and capillary transfer in 20x SSC (see Appendix 2.8) of RNA onto the membrane allowed to proceed overnight. Thereafter the membrane was removed,

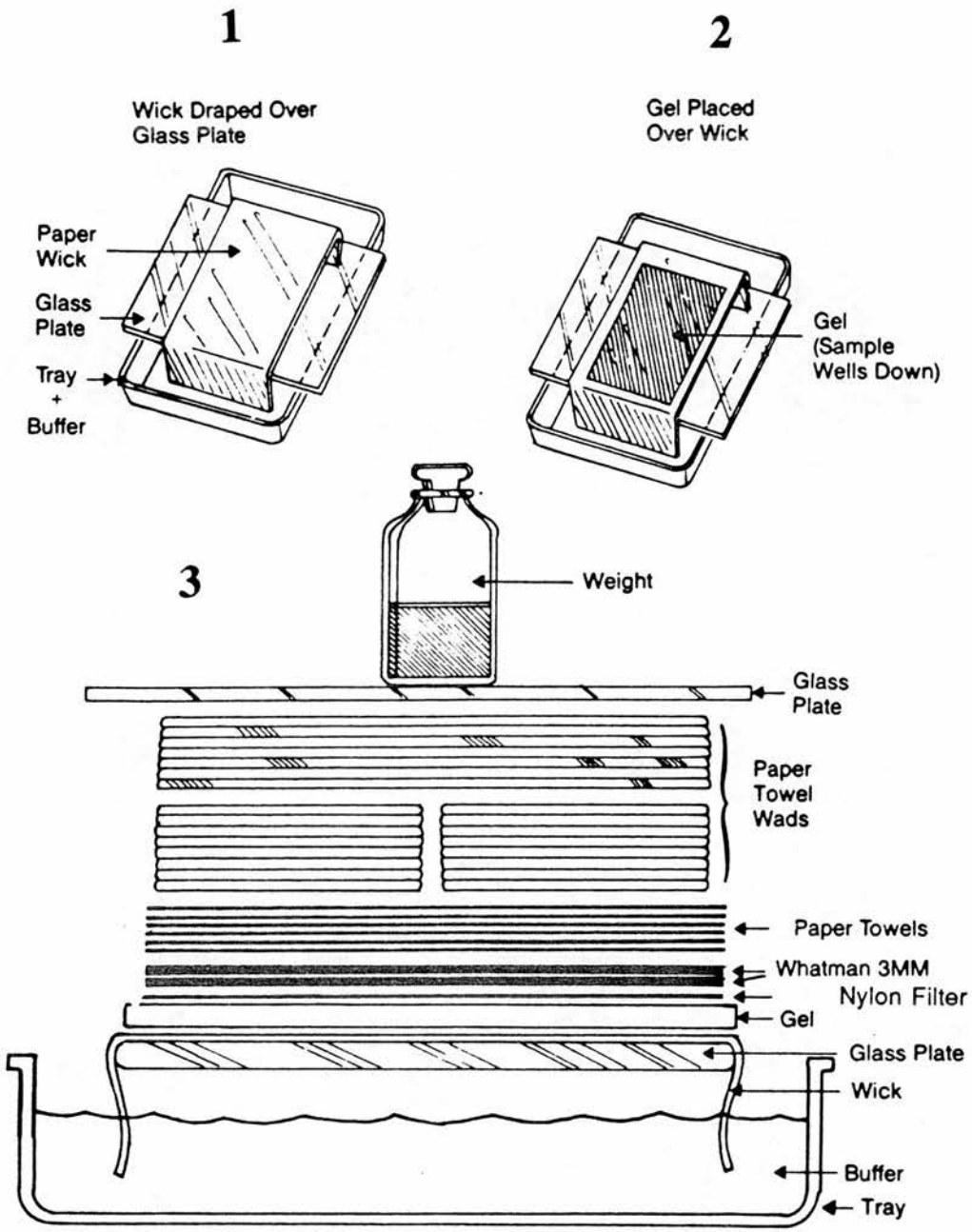

Figure 7.1 Northern blot transfer set up diagram. (1) Placing the Whatman 3MM paper as a wick over glass plate. (2) Mounting the gel on wick. (3) Schematic diagram of final layered organization of materials. (Modified from Davis et al , 1986).

wrapped in plastic film, exposed to UV light (260 nm) for 5 min to permanently fix the RNA and stored until the hybridization was performed.

Preparation of the radiolabelled probes

Human inhibin α-cDNA, 1160 bp in length, was provided by Dr. R.G. Forage. The DNA fragments were released from pBR322 by Pst I and further purified on a NuSieve gel run in TAE buffer. The purified DNA was retrieved by glass powder elution using the GENECLEAN II Kit (BIO 101 Inc.). This preparation was done by Dr. Philippa Saunders and Dr. Kogie Reddi.

Probe labelling

^{32}P-inhibin α-cDNA probe

A labelled cDNA probe was prepared from the DNA obtained above by the "random primed" DNA labelling method (Feinberg & Vogelstein,1983; 1984) using a random primed DNA labelling kit (Boehringer Mannheim). Briefly, 9 µl purified cDNA solution (50 ng) in a 1.5 ml microcentrifuge tube was heated at 95°C for 5 min to denature the DNA and then immediately placed on ice. The following solutions were added in order; 2 µl of reaction mixture (containing 100 µl of hexanucleotide mixture in 10x reaction buffer), 1 µl dATP, 1 µl dGTP, 1 µl dTTP, 5 µl of ^{32}P-dCTP (Amersham, 10 µCi/µl) and 1 µl Klenow polymerase (2 U/µl). The reaction mixture was incubated at 37°C for 30 min. The synthesized double-stranded DNA probe was denatured by adding 50 µl of 10 N NaOH and incubating for a further 5 min. The reaction was stopped by adding 600 µl of 1 M Tris-HCl (pH 7.6) and 375 µl of 0.1 N HCl. The probe was diluted in hybridization buffer and incubated with the membrane (see below).

^{32}P-inhibin βB-subunit riboprobe

The radioactive probe was synthesized from cDNA by DNA-independent RNA polymerase transcription using an RNA transcription kit (Stratagene). 8 µl of linearized plasmid DNA (1 µg), 1µl RNAse inhibitor, 1 µl rATP(10 mM), 1 µl rCTP (10 mM), 1 µl rGTP (10 mM), 5 µl of 5x transcription buffer, 1 µl 0.75 M dithiothreitol (DTT, Sigma), 5 µl ^{32}P-UTP (10 µCi/µl, Amersham) and 2 µl of T7 RNA polymerase (1 U) were added in order to a 1.5 microcentrifuge tube. The reaction mixture was incubated at 37°C for 1h and made up to 100 µl with 75 µl of TE buffer (0.1M triethanolamine, pH 8.0). The mixture was extracted twice with an equal

volume of phenol/chloroform (see Appendices 2.9 and 2.10). The RNA was precipitated by adding 10 µl of 3 M sodium acetate and 330 µl cold ethanol at -70°C for 3 h. The RNA was collected by centrifugation at 13,000 g for 15 min at 4°C. The RNA pellet was washed in 400 µl 75% cold ethanol, air-dried and dissolved in 50 µl of sterile water. The activity of the probe was determined by counting in a scintillation counter (1µl of the probe to 5 ml of scintillation fluid).

Hybridization

Inhibin α-subunit cDNA probe

The membrane was placed in a hybridization bottle (Hybraid) and prehybridized with 50 ml hybridization buffer containing 0.2 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 1% BSA (Sigma), 7% SDS and 15% deionised formamide at 65°C overnight with gentle rotation. The hybridization was performed by adding the prepared probe into the bottle to obtain a final concentration of 0.5 x 10^6 cpm/ml and continued at 65°C for a further 16 h. The filter was rinsed three times with prewarmed (65°C) washing buffer (40 mM sodium phosphate pH 7.2, 1 mM EDTA and 0.1% SDS), washed thoroughly by rotation (two changes of buffer) at 65°C for 30 min and finally rinsed in prewarmed buffer. The filter was wrapped in plastic film and exposed to Kodak Xomat AR-5 film in an X-Ray film holder cassette containing an image intensification screen (Du Pont Cronex) for 3 days at -70°C. The exposed film was developed in Kodak developer for 2 min, rinsed in water for 20 sec and then fixed in 1 in 4 diluted Kodak Unifix for 2 min.

Inhibin βB-subunit riboprobe

The α-inhibin ^{32}P-cDNA probe was stripped from the membrane by washing with 0.2% SDS in sterile water at 90°C for 15 min. The filter was rehybridized with ^{32}P-βB-subunit riboprobe as described for the cDNA probe except the hybridization buffer was replaced with high-stringency formula hybridization buffer (0.2 M sodium phosphate pH 7.2, 1 mM EDTA, 1% BSA, 7%SDS and 45% deionized formamide) and the final concentration of the probe was 2x10^6 cpm/ml.

The evenness of loading of RNA was assessed by Dr. Kogie Reddi by reprobing the membrane with a labelled oligonucleotide directed against 18S RNA.

7.2.2 Results

Expression of inhibin α-subunit m-RNA

Total RNAs from 7 weeks (E), 16 weeks (M) and term (L) placentae had mRNA which hybridized to an inhibin α-subunit cDNA probe (Figure 7.2). There was some background hybridization to both ribosomal RNA species. The specificity of the hybridization signal obtained and the apparent message size was verified by studying the signal obtained with 5 μg of messenger RNA (A) separated on the same agarose gel. The results showed expression of inhibin α-subunit mRNA throughout pregnancy. The message size was approximately 1.6 kb (Figure 7.2 arrowed). Expression was high in early pregnancy (7 weeks) when compared to other stages of pregnancy. The signal was especially low in mid-term (16 weeks).

Expression of inhibin βB-subunit m-RNA

Inhibin βB-subunit riboprobe demonstrated specific hybridization to both total RNA and mRNA and revealed only one message size of approximately 2.4 kb throughout pregnancy (Figure 7.3). However, mRNA expression appeared to vary at different stages of pregnancy. The highest level of expression was observed in the sample obtained at 16 weeks and slightly less at term while the expression was least in early (7 weeks) pregnancy.

7.2.3 Discussion

Previous studies have demonstrated expression of inhibin α- and β-subunit-genes in human term placenta (Mayo et al, 1986; Davis et al, 1987; Reddi et al, 1990b). The results obtained from this study confirm the expression of inhibin α- and βB-subunit genes in term placenta and the expression of these two inhibin-subunit genes throughout pregnancy. The message size of these subunits are approximately the same as previously reported (Davis et al, 1987; Reddi et al, 1990b).

In other species, reproductive tissues such as follicles and corpora lutea have been shown to demonstrate different expression of inhibin genes during growth and development (Meunier et al, 1988a; Woodruff et al, 1988; Torney et al, 1989; Schwall et al, 1990). The results from this study also demonstrate differential expression of inhibin α-and βB-subunit mRNAs in human placenta at different stages of pregnancy.

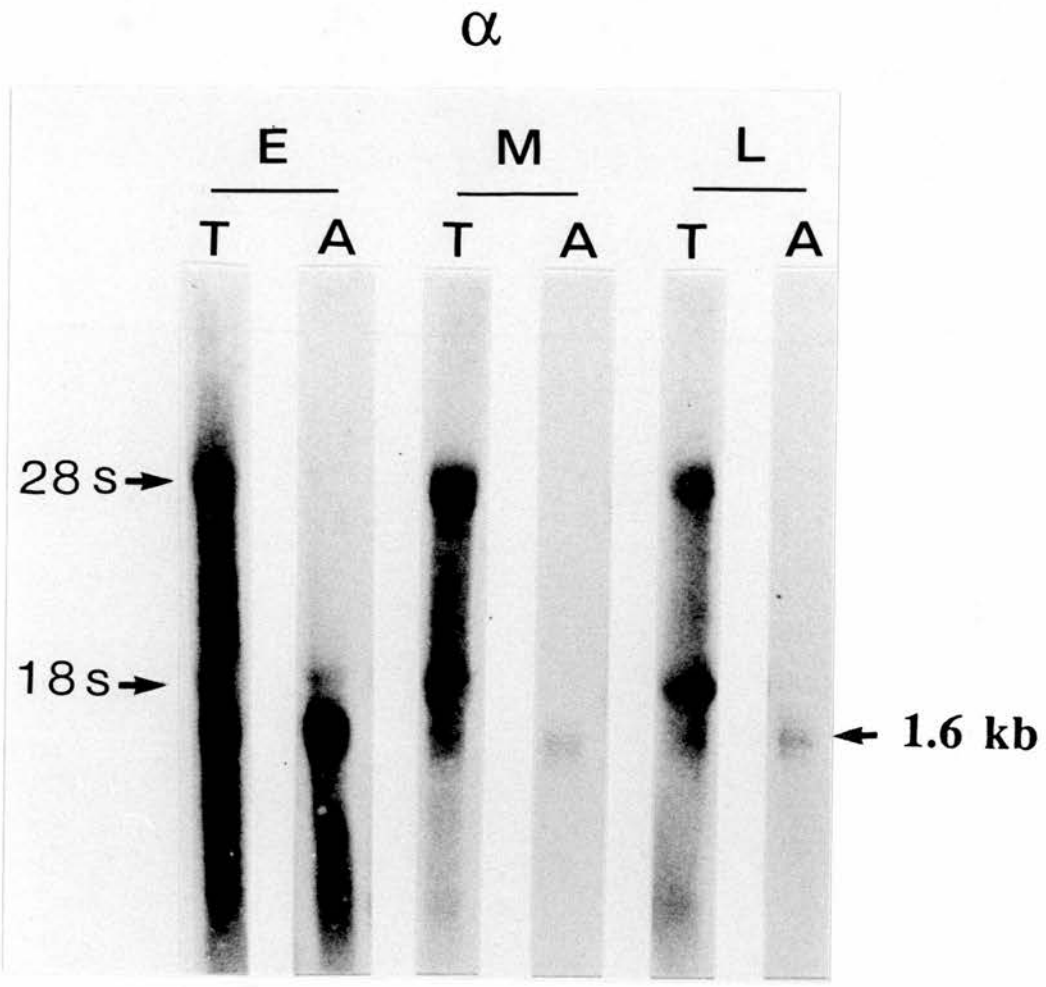

Figure 7.2 Northern blot analysis of inhibin α-subunit mRNA in placental tissues from 7 weeks (E), 16 weeks (M) and term (L) pregnancy. 20 μg of total RNA (T) and 5 μg of mRNA (A) from each stage of pregnancy showed hybridization to the inhibin α-subunit cDNA probe. The specific hybridization to mRNA demonstrated the message size of inhibin α-subunit mRNA as 1.6 kb. The signal was highest at 7 weeks and hardly detected at latter stages of pregnancy.

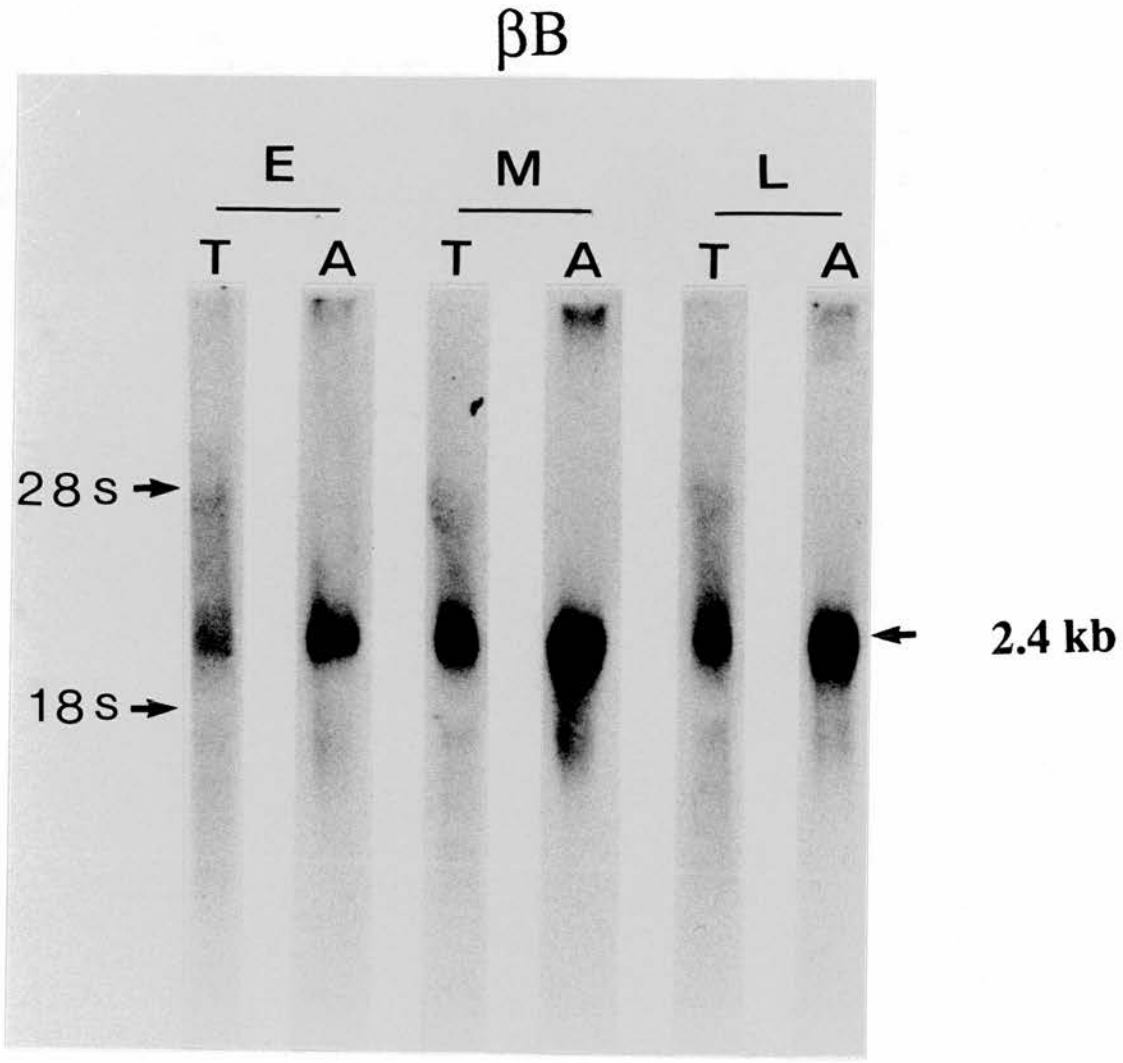

Figure 7.3 Northern blot analysis of inhibin βB-subunit mRNA in placental tissues from 7 weeks (E), 16 weeks (M) and term (L) pregnancy. 20 μg of total RNA (T) and 5 μg of mRNA (A) from each stage of pregnancy showed specific hybridization to the inhibin βB-subunit riboprobe. The specific hybridization to total RNA and mRNA corresponded to a message size of inhibin βB-subunit mRNA of 2.4 kb. The signal was highest at mid-term, and least but easily detectable at 7 weeks of pregnancy.

Figure 7.2 illustrates that inhibin α-subunit gene expression is greatest in the early stage of pregnancy (7 weeks) but very much less in mid-term (16 weeks) and term. This would suggest that, during pregnancy, the placenta might produce different amounts of inhibin α-subunit protein throughout gestation. This result agrees with the finding outlined in Chapter 4; that inhibin immunoactivity in placenta extracts is abundant in early pregnancy in comparison with mid-term and term.

The expression of inhibin βB-subunit mRNA has been also found to vary during pregnancy (Figure 7.3). The message is abundant throughout pregnancy but the expression of this subunit gene in mid-term and term is greater than in early pregnancy. This finding supports the results from the immunoneutralization of inhibin bioactivity in Chapter 4 which suggest that the placenta may produce activin in mid-term and term pregnancy.

The present study has demonstrated the expression of inhibin α- and β-subunit mRNAs in placental tissue throughout pregnancy. The results presented elsewhere in this thesis have shown the localization of inhibin subunit proteins in trophoblast cells and also the measurement of inhibin *in vitro* bioactivity in placental extracts throughout pregnancy. These results taken together provide conclusive evidence that the placenta is a source of inhibin and activin during pregnancy.

CHAPTER 8

Cellular localization of inhibin-subunit mRNAs in human placental tissue from early and term pregnancy by *in situ* hybridization

8.1 Introduction

The development of *in situ* hybridization methods have allowed us to investigate expression of the inhibin gene in different cell types of gonadal and non-gonadal tissues in various species. We expect data from this method to aid us in understanding the physiology of inhibin at the cellular level.

The presence of, and changes in, inhibin α-, βA- and βB-subunit mRNA expression in rat ovarian cell types during the oestrous cycle have been shown by Meunier et al (1988a) and Woodruff et al (1988). Inhibin gene expression during the rat oestrous cycle is modulated by LH and FSH (Rivier et al, 1989). In cow ovaries, inhibin α-subunit mRNA is expressed in granulosa and thecal cells of antral follicles while only βA-subunit mRNA is localized in granulosa cells (Torney et al, 1989). In the monkey, the expression of inhibin subunit mRNA is differentially regulated during the growth and development of ovarian follicles. The βB-subunit mRNA is the only subunit that is expressed in granulosa cells of small antral follicles while inhibin α- and βA-subunit mRNAs are expressed in dominant follicles (Schwall et al, 1990).

In the human placenta, the available data concerning inhibin/activin gene expression has only been obtained from Northern blot analysis of term placental RNA (Davis et al, 1987; Reddi et al, 1990b). The cellular localization of inhibin subunit proteins in both cytotrophoblast and syncytiotrophoblast cells by immunohistochemistry has been reported in Chapter 6. However, gene expression and protein localization may not necessarily be within the same cell types. Therefore, to further our understanding of the site of inhibin subunit protein synthesis and gene expression, the study described in this chapter was carried out to localize inhibin mRNA in human placental tissue by *in situ* hybridization.

8.2 Cellular localization of inhibin α-, βA- and βB-subunit mRNAs in human placenta

In situ hybridization of inhibin-subunit mRNAs was performed using the method described as follows.

8.2.1 Materials and Methods

8.2.1.1 Tissue preparation

Human placental tissues were obtained from pregnancy termination at 7 weeks and term. The tissues were fixed using the method outlined in Chapter 6, section 6.3.1.1. Approval of the Local Ethical Committee (Division of Reproductive Medicine, Lothian Health Board) was obtained. The placentae were cut across the basal plate about 5 mm-thick, and washed in PBS to remove blood. The tissues were transferred to freshly prepared ice-cold 4% paraformaldehyde in PBS, pH 7.4, and fixed at 4°C overnight and then dehydrated and embedded in paraffin. Sections, 4 μm thick, were cut and mounted on tespa-coated slides (see Appendix 3.1) then incubated at 60°C overnight.

8.2.1.2 Preparation of ^{35}S-labelled RNA probes

Nucleotide sequences of the human α-, βA- and βB-inhibin subunit cDNAs (Mason et al, 1986) were studied to find areas of sequences which were not homologous. Oligo nucleotide primers with restriction sites at their 5' ends were synthesized and used to amplify the selected sequences of cDNA by Polymerase chain reaction (PCR) technique from inhibin α, βA- and βB-subunit cDNAs provided by Dr. R. G. Forage (Forage et al, 1986; Reddi et al, unpublished data). The cDNA obtained for each subunit was purified and subcloned into precut pBluescript SK(/-) plasmid; plasmids were transformed into competent XL I blue *E. coli*, amplified and sequenced to confirm correct cDNA insertion (Saunders, unpublished data). The details of the cDNA fragments thus obtained are described as follows:

The α-subunit cDNA was 345 base pairs in length, corresponding to the nucleotides 712-1057, coding part of the mature region of human inhibin α-subunit. The βA-subunit cDNA length was 447 base pairs, complementary to nucleotides 670 -1118, corresponding to amino acids in the pro- and mature regions of the βA-subunit protein precursor. The βB-subunit cDNA was 558 base pairs in length, corresponding to the

nucleotides 745-1303, coding part of the inhibin βB-subunit mature region and 3' untranslated region.

Plasmids were amplified, extracted, and purified prior to use as templates for cRNA synthesis using the Mini-Prep Kit Plus (Pharmacia) with slight modifications as follows:

Plasmid amplification, extraction and purification

To 15 ml of Terrific Broth (Sambrook et al, 1989a; see Appendix 3.2), containing ampicillin (50 μg/ml, Beecham Research Laboratories, Brentford), a single bacterial colony was inoculated and incubated overnight at 37°C with shaking. Cells were then harvested by centrifugation at 2,000 g for 10 min. The supernatant was discarded and the pellet resuspended by vortexing vigorously in 1 ml of lysis buffer containing 50mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA and 0.15% Kathon CG/ICP (Mini-Prep Kit Plus, Pharmacia). The reaction mixture was incubated on ice for 5 min, 2 ml of 0.2 M NaOH with 1% Triton X-100 was added, mixed by inverting several times and incubated for a further 5 min on ice. The reaction mixture was neutralized by adding 1.5 ml of 3M sodium acetate (pH 4.8), mixed by inverting and left on ice for 5 min. The *E.coli* chromosomal DNA and cell wall debris were precipitated by centrifugation at 12,000 g at 4°C for 10 min. Four aliquots of 750 μl supernatant were carefully transferred to 1.5 ml microcentrifuge tubes containing 750 μl of isopropanol (Sigma). The mixture was mixed by inverting and incubated at room temperature for 10 min and the DNA was precipitated at 12,000 g for 5 min at 4°C. The supernatant was discarded and the DNA pellet washed with 750 μl of isopropanol without being disturbed and allowed to air-dry before being dissolved in 50 μl of TE buffer (column buffer) containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The extracted DNA was purified using spun columns (Pharmacia) following the supplier's instructions (see Appendix 3.3). Briefly, 50 μl of extracted DNA was carefully applied to the centre of the surface of the compacted gel bed. The column was centrifuged at 400g for 2 min, removed and discarded while approximately 50 μl of the effluent which contained the purified DNA was collected. The amount of purified DNA in the effluent was estimated using ethidium bromide fluorescence quantitation (Maniatis et al, 1982; see Appendix 3.4)

Plasmid linearization and purification

The plasmids containing inhibin α- and βA-subunit cDNAs were linearized with BamH I and Xho I to obtain the antisense and sense probes respectively while the βB-subunit cDNA plasmid was linearized with Hind III and EcoR I for the antisense and sense probes. Briefly, 10 µl of purified plasmid (approximately 750 ng-1 µg), 2 µl of 10x restriction buffer, 1 unit of the appropriate restriction enzyme (Boehringer Mannheim) and 7 µl of sterile water were pipetted in sequence to a microcentrifuge tube and the reaction mixture incubated at 37°C for 1.5 h. After adding 1µl of proteinase K type XXVIII (Sigma, 10mg/ml in water), the mixture was incubated for a further 30 min at 37°C. The volume of the mixture was made up to 100 µl with 80 µl of sterile water and extracted with an equal volume of phenol/chloroform. The phenol/chloroform layer was discarded and re-extracted with an equal volume of chloroform (IBI). DNA was precipitated following the addition of 200 µl of cold ethanol (BDH) and 20 µl of 10.5 M ammonium acetate (Sigma) by incubation at -20°C for 2 h. DNA was recovered by centrifugation at 13,000 g for 15 min. The pellet was washed with 400 µl of 75% ethanol, air-dried, resuspended in 10 µl of TE buffer and stored at -20°C.

To determine the complete linearization and the amount of DNA in the preparation, 5 µl of a mixture of 1 µl of linearized or uncut DNA, 2 µl of "orange juice" (see Appendix 3.5) and 7 µl of water was loaded on to a 0.8% agarose gel (see Appendix 3.6) along with known concentrations (10-100 ng/µl) of a standard-size marker (pBR322 DNA standard, Pharmacia). The gel was run at 100 V for approximately 3 h, then visualized and photographed under a UV transilluminator.

Probe labelling

^{35}S-inhibin subunit sense and antisense riboprobes were generated by transcription with the DNA-dependent RNA polymerase labelling method using an RNA transcription kit (Stratagene). The transcription reaction was performed by adding the following solutions into a 1.5 ml microcentrifuge tube in order; 6 µl 5x transcription buffer, 1 µl 10 mM rCTP, 1 µl 10 mM rATP, 1 µl 10 mM rGTP, 1 µl 1M dithiothreitol (DTT, Sigma), 3 µl sterile water, 12 µl ^{35}S-rUTP (10 µCi/µl, Amersham), 5 µl of restricted DNA template (0.5-1 µg/5 µl), 1.2 µl of RNase-Block II (1U/µl) and 0.8 µl of T3 or T7 RNA polymerase (0.8 U). The reaction mixture was incubated at 37 °C for 25 min. 0.8 µl RNA polymerase was then added and the

reaction continued at 37°C for a further 25 min. The activity of incorporated nucleotides was monitored by TCA precipitation of 1 µl of the reaction mixture at this step (Sambrook et al, 1989b). The reaction was stopped by adding 2 µl tRNA (10mg/ml, Sigma) and 1 µl RNase-free DNase I (1mg/ml, Promega) and incubated at 37°C for 10 min. After adding 1 µl of 200 mM EDTA and 67.5 µl of TE buffer containing 50 mM DTT the mixture was extracted twice with 100 µl of phenol and once with phenol:chloroform (1:1) and precipitated with 10 µl 3 M sodium acetate and 330 µl cold ethanol at -20°C overnight. The RNA was pelleted by centrifugation at 13,000 g for 15 min. The RNA pellet was washed twice in cold 80% ethanol containing 50 mM DTT and once in cold ethanol. The pellet was air-dried and dissolved in 100 µl of DEPC-treated water (see Appendix 3.7) containing 50 mM DTT.

Probe hydrolysis

To obtain shorter RNA probes approximately 100 bases in length which give higher hybridization signals, the cRNAs obtained above were subjected to alkaline hydrolysis. 100 µl of dissolved RNA pellets from the previous step were added to an equal volume of freshly prepared 0.2 M carbonate buffer containing 80 mM sodium bicarbonate, 120 mM sodium carbonate and 50 mM DTT. The reaction mixture was incubated at 60°C for 30 min. The reaction was stopped by the addition of 100 µl of 6M ammonium acetate (pH 5.2) and 1 µl of tRNA (10 mg/ml). The RNA was precipitated with three volumes of cold ethanol at -20°C overnight. The pellet was collected by centrifugation at 13,000 g for 20 min. The pellet was washed twice in cold 80% ethanol containing 50 mM DTT and once in cold ethanol. After air-drying, the pellet was dissolved in 20 µl of DEPC-treated water containing 50 mM DTT and the activity of the probe was monitored by TCA precipitation of 1 µl of the solution as outlined above. The probe was kept at -70°C until the hybridization was performed.

8.2.1.3 *In situ* hybridization procedure

The *in situ* hybridization procedure was performed as described by Wilkinson et al (1987a) in combination with the high-stringency washing steps described by Wilkinson et al (1987b).

Prehybridization treatment

The sections were deparaffinized twice in xylene (BDH) for 5 min and rehydrated through a series of alcohols (100, 95, 85, 70, 50 and 30% EtOH) for 2 min each. After further rehydration for 5min each in normal saline and PBS the sections were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 20 min and then washed twice in PBS (5 min each). The sections were treated for 7.5 min with 20 µg/ml proteinase K (Sigma) in buffer (pH 7.2) containing 50 mM Tris and 5 mM EDTA to allow permeability of the probes. After washing for 5 min in PBS the sections were refixed in 4% paraformaldehyde in PBS for 10 min, then immersed in sterile water and TE buffer for 10 and 30 sec respectively. To reduce electrostatic binding of the probe the sections were incubated twice in 0.25% acetic anhydride (Sigma) in TE buffer for 10 min (due to the very short half-life of acetic anhydride in aqueous solution the sections must be placed in a staining dish containing TE buffer and a magnetic bar and placed on a magnetic stirrer before acetic anhydride is added). The sections were washed in PBS and 0.85% NaCl for 5 min each, then dehydrated through graded EtOH (30, 50, 70, 85, 95%) for 1 min each. After dehydration twice in 100% EtOH for 5 min the sections were air-dried and the hybridization performed.

Hybridization

The ^{35}S-riboprobes were diluted in hybridization mixture (see Appendix 3.8) to obtain $1x10^5$ dpm/µl. The diluted probes were heated at 80°C for 2 min and cooled on ice before applying to the sections (10 µl/section). After overlaying with tespa-coated cover slips the sections were hybridized overnight in a humidified chamber (saturated with 5x SSC and 50% formamide) at 55°C.

Posthybridization treatment

The sections were washed with 5x SSC containing 10 mM DTT for 30 min at 55°C and then with 2x SSC, 50% formamide and 0.1 M DTT for 20 min at 65°C. The sections were washed three times in buffer (pH 7.5) containing 0.5 M NaCl, 10 mM Tris and 5 mM EDTA (NTE) for 10 min at 37°C and were then treated with RNase (40 µg/ml) in NTE for 30 min at 37°C and washed in NTE for 15 min at the same temperature. The washing step with 2x SSC, 50% formamide and 0.1 M DTT for 20 min at 65°C was repeated, then the sections were washed in 2x SSC three times (10 min each) and finally washed thrice in 0.1x SSC (10 min each) at room temperature.

The sections were dehydrated in graded EtOH (30, 50, 70, 90%) containing 0.3 M ammonium acetate for 1 min each and then in 100% EtOH twice for 5 min before air-drying.

Autoradiograph dipping

This step was performed in a dark room with a safety light. The slides were dipped in a mixture of Ilford K5 emulsion in water (1:1, diluted immediately before use) at 40-42°C twice and left to drain. The slides were then placed in a light-tight box and left to dry for 3 h or overnight before being left to autoexpose in a light-tight box, containing silica gel, wrapped in aluminum foil for 2-4 weeks at 4°C.

Autoradiograph developing

This step was also carried out in a dark room. The slides were placed in developer D 19 (Kodak) for 4 min and then rinsed in distilled water before being placed in fixative (Kodafix:distilled water, 1:3) for 4 min and washed twice in distilled water for 10 min. The slides were counterstained in 1% Methyl Green in water (Sigma) for 2 min and mounted in DPX (Sigma) before visualizing under the light microscope.

8.2.2 Results

The α-subunit riboprobe was hybridized to sections of 7 week and term placentae (Figures 8.1, 8.2, 8.3 and 8.4). In the area of the chorionic villi, inhibin α-subunit messenger RNA expression was localized only within trophoblast cells. High magnification photomicrographs (Figures 8.2 and 8.4) clearly showed that both syncytiotrophoblast and cytotrophoblast cells expressed messenger RNA (silver grains). The amount of inhibin α-subunit mRNA expression in 7 week placental tissue was higher than in term placenta. The βA-subunit riboprobe also hybridized to 7 week and term placental tissue and the expression of mRNA was confined to trophoblast cells (Figures 8.5, 8.6, 8.7 and 8.8). In 7 week placenta, the high magnification photomicrograph (Figure 8.6) shows hybridization of the βA-subunit riboprobe occurred on syncytiotrophoblast and cytotrophoblast cells, and the expression of this inhibin subunit mRNA appeared stronger in trophoblast cells of term placental tissue (Figure 8.8). None of the placental tissues from 7 weeks and term pregnancy demonstrated hybridization to sense inhibin α- and βA-subunit riboprobes. The inhibin βB-subunit riboprobe hybridized to trophoblast cells of 7 week placental tissue

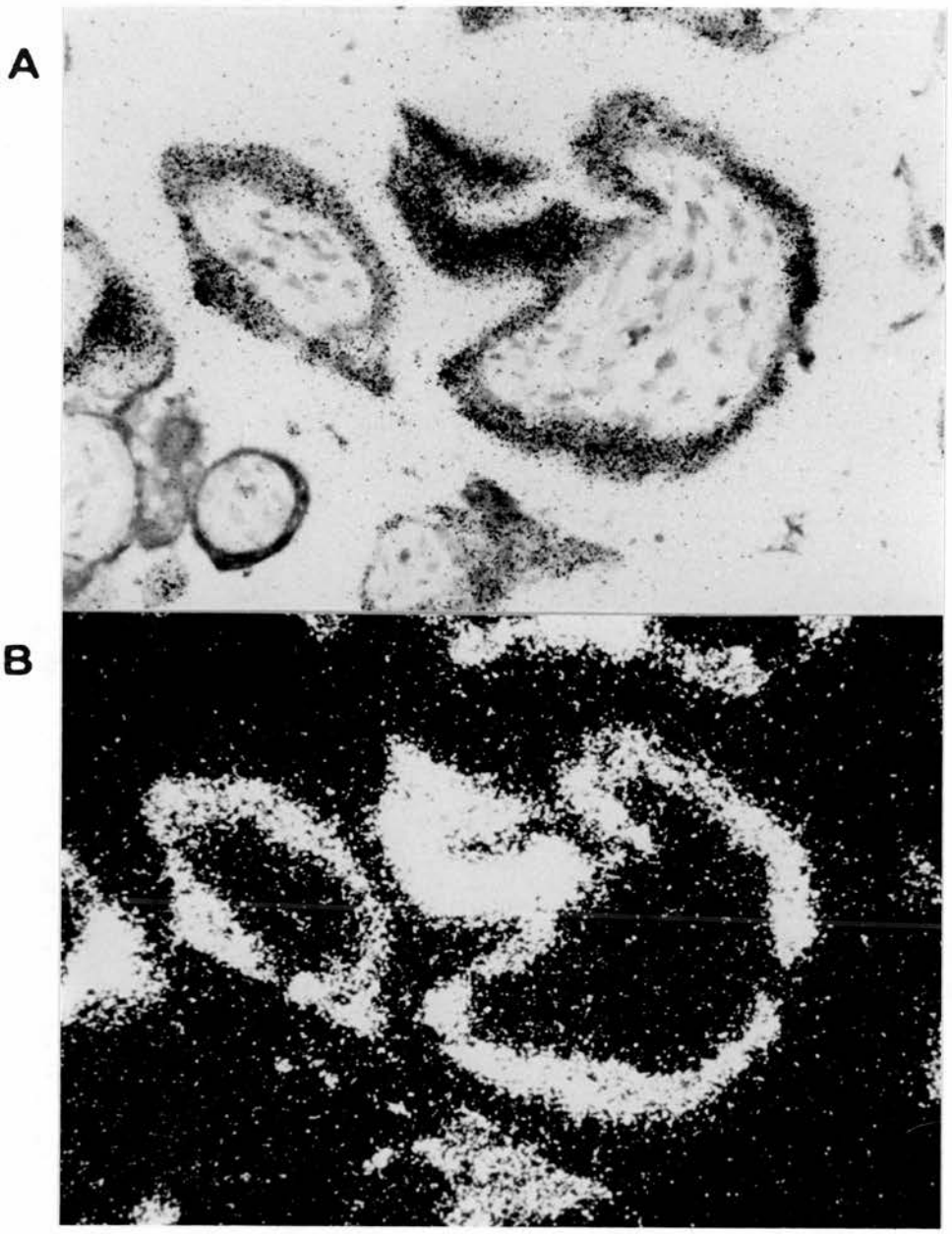

Figure 8.1 Hybridization of 7 weeks placental tissue with inhibin α-subunit riboprobe. Low-magnification (x20) bright field photomicrograph (A) and dark field photomicrograph (B) of the same area showing expression of inhibin α-mRNA (silver grains) which appears to be within trophoblast cells.

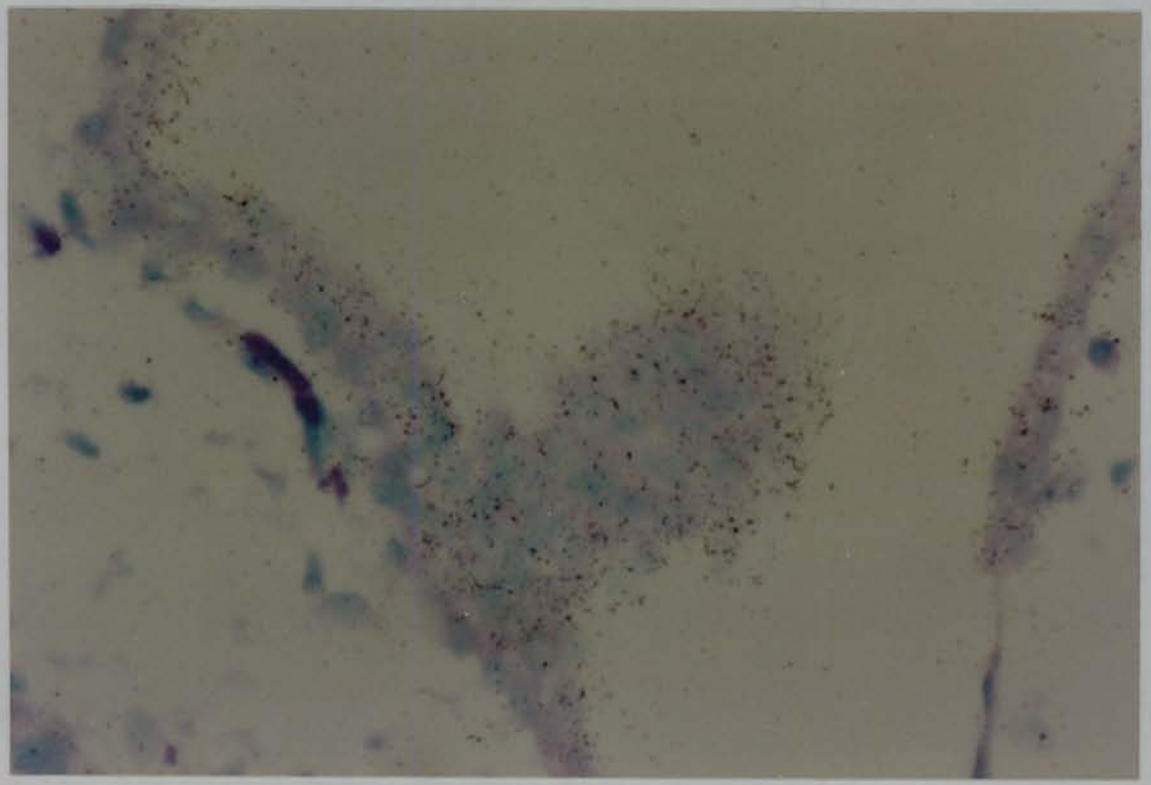

Figure 8.2 High-magnification photomicrograph (x40) demonstrating hybridization of the inhibin α-subunit probe in syncytiotrophoblast and cytotrophoblast cells of 7 week placental tissue.

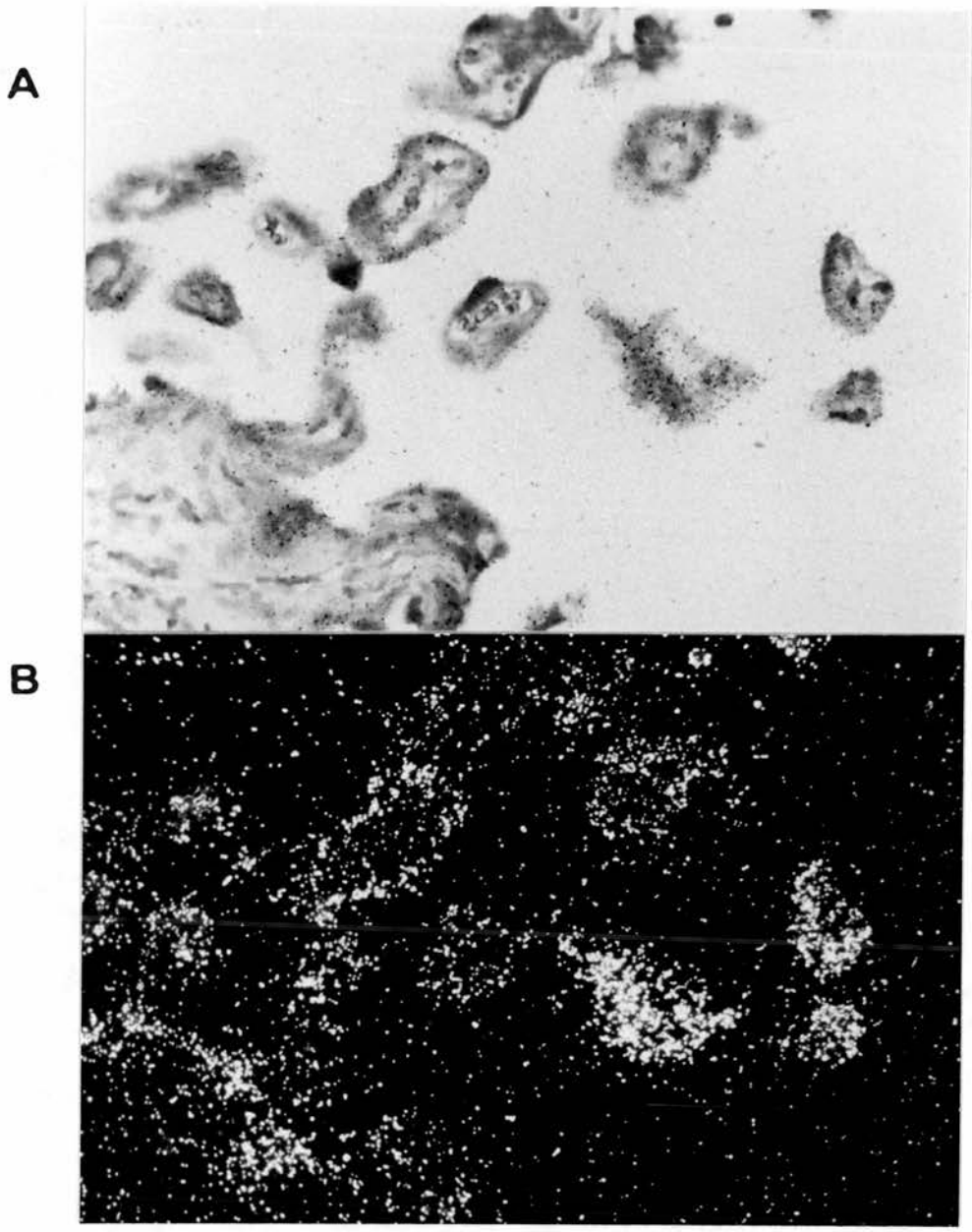

Figure 8.3 Hybridization of term placental tissue with inhibin α-subunit riboprobe. Low-magnification (x20) bright field photomicrograph (A) and dark field photomicrograph (B) of the same area showing expression of inhibin α-mRNA within trophoblast cells.

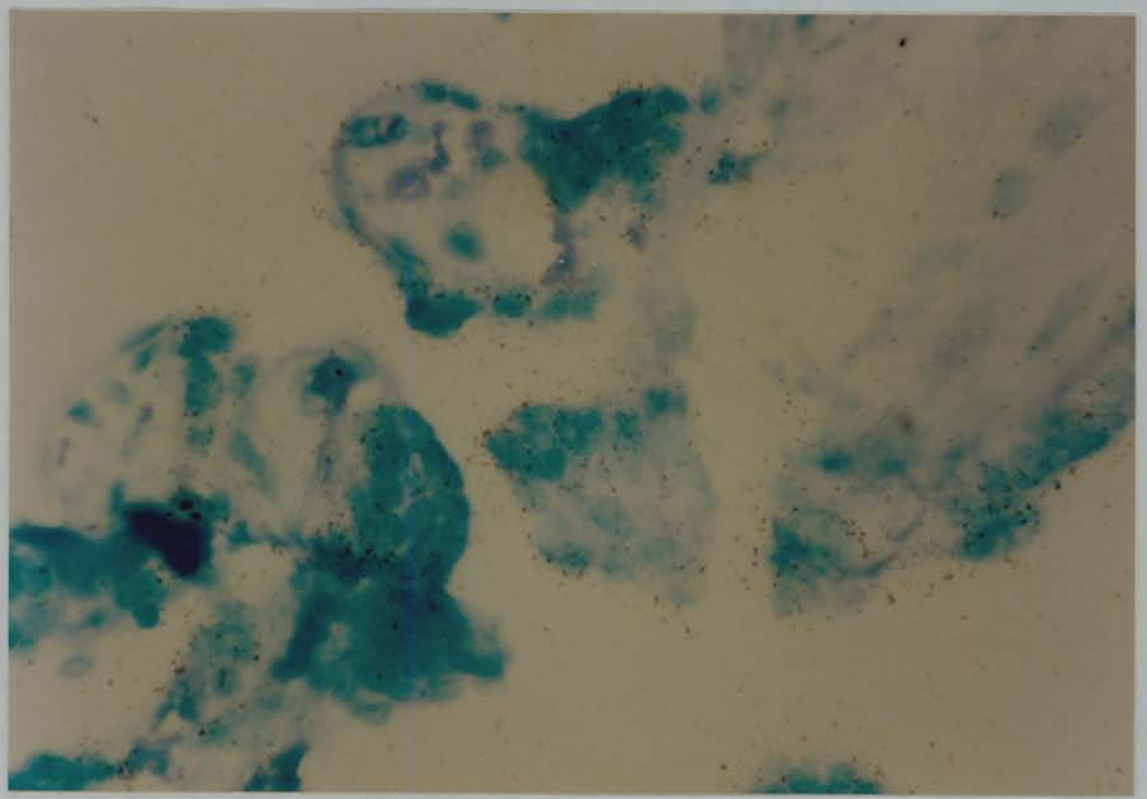

Figure 8.4 High-magnification photomicrograph (x40) demonstrating hybridization of the inhibin α-subunit probe in trophoblast cells of term placental tissue.

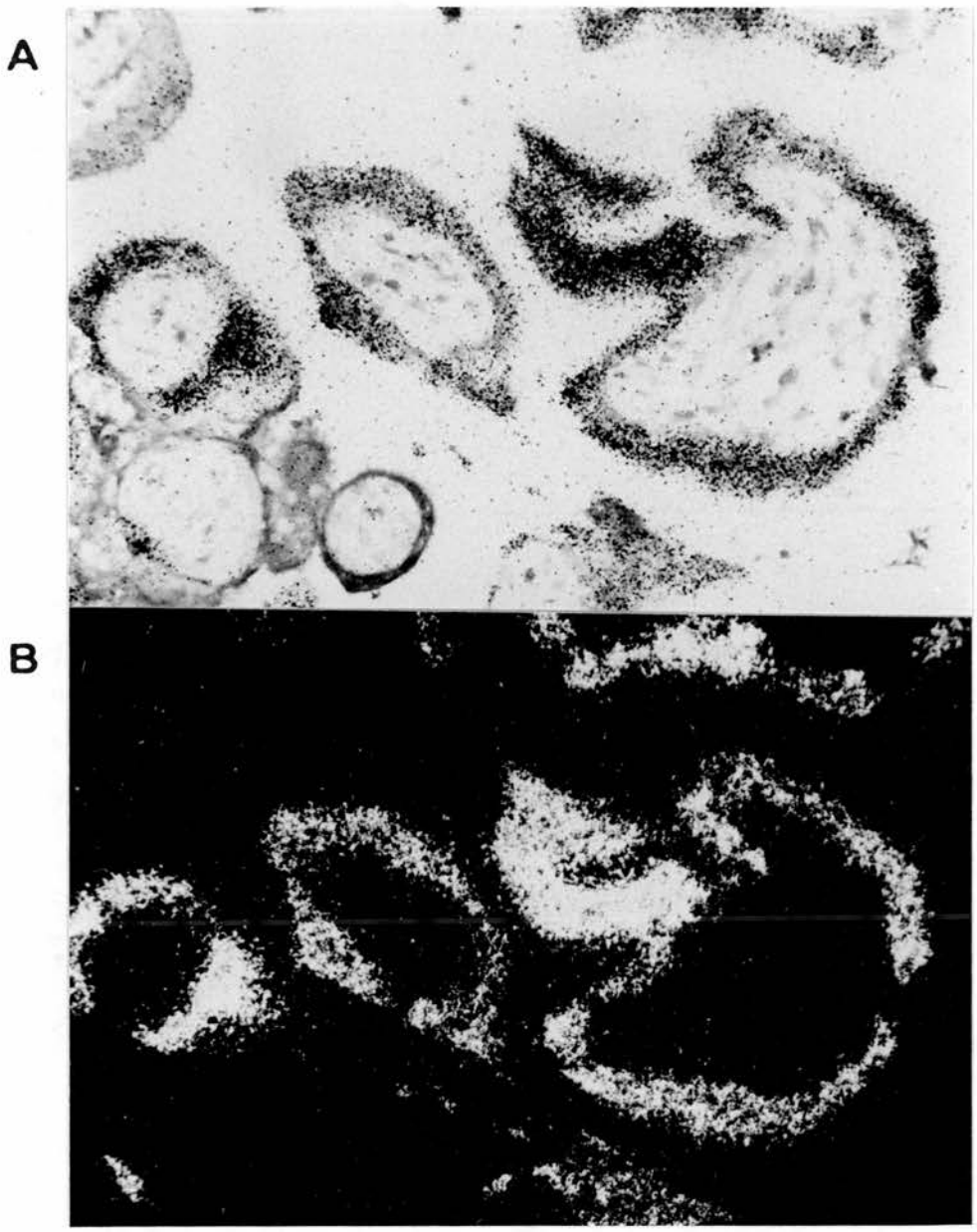

Figure 8.5 Hybridization of 7 weeks placental tissue with inhibin βA-subunit riboprobe. Low-magnification (x20) bright field photomicrograph (A) and dark field photomicrograph (B) of the same area showing expression of inhibin βA-mRNA within trophoblast cells.

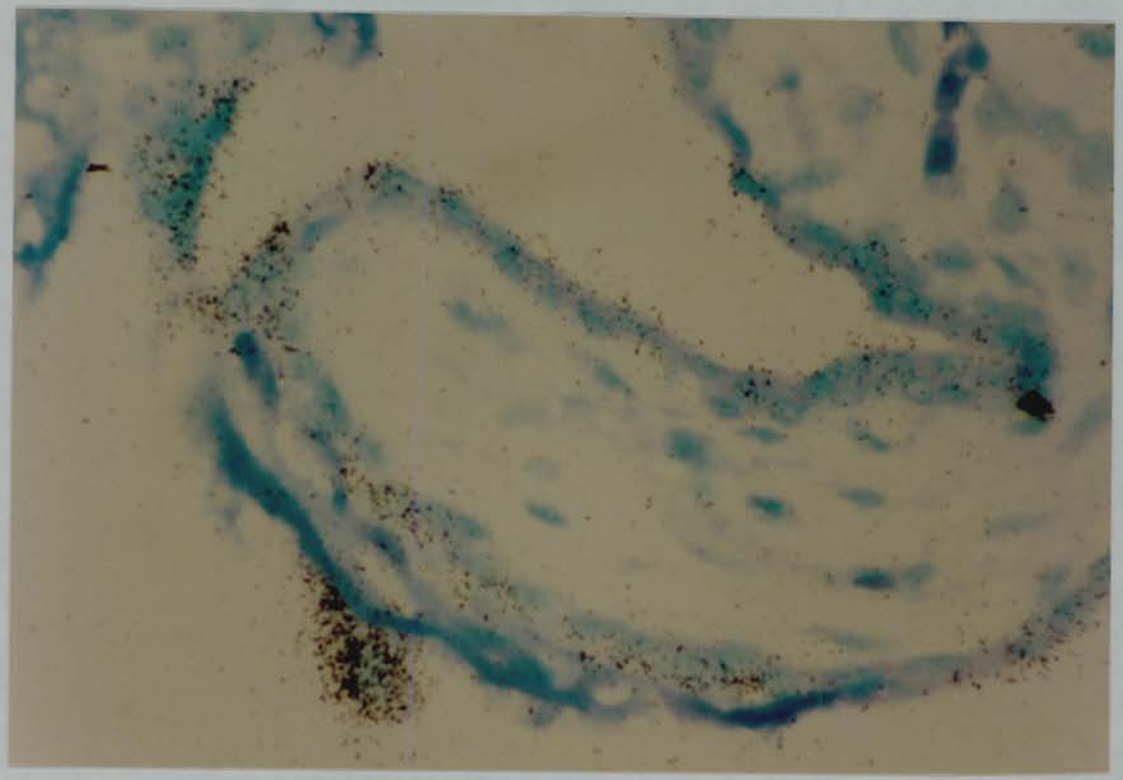

Figure 8.6 High-magnification photomicrograph (x40) demonstrating hybridization of the inhibin βA-subunit riboprobe in syncytiotrophoblast and cytotrophoblast cells of 7 week placental tissue.

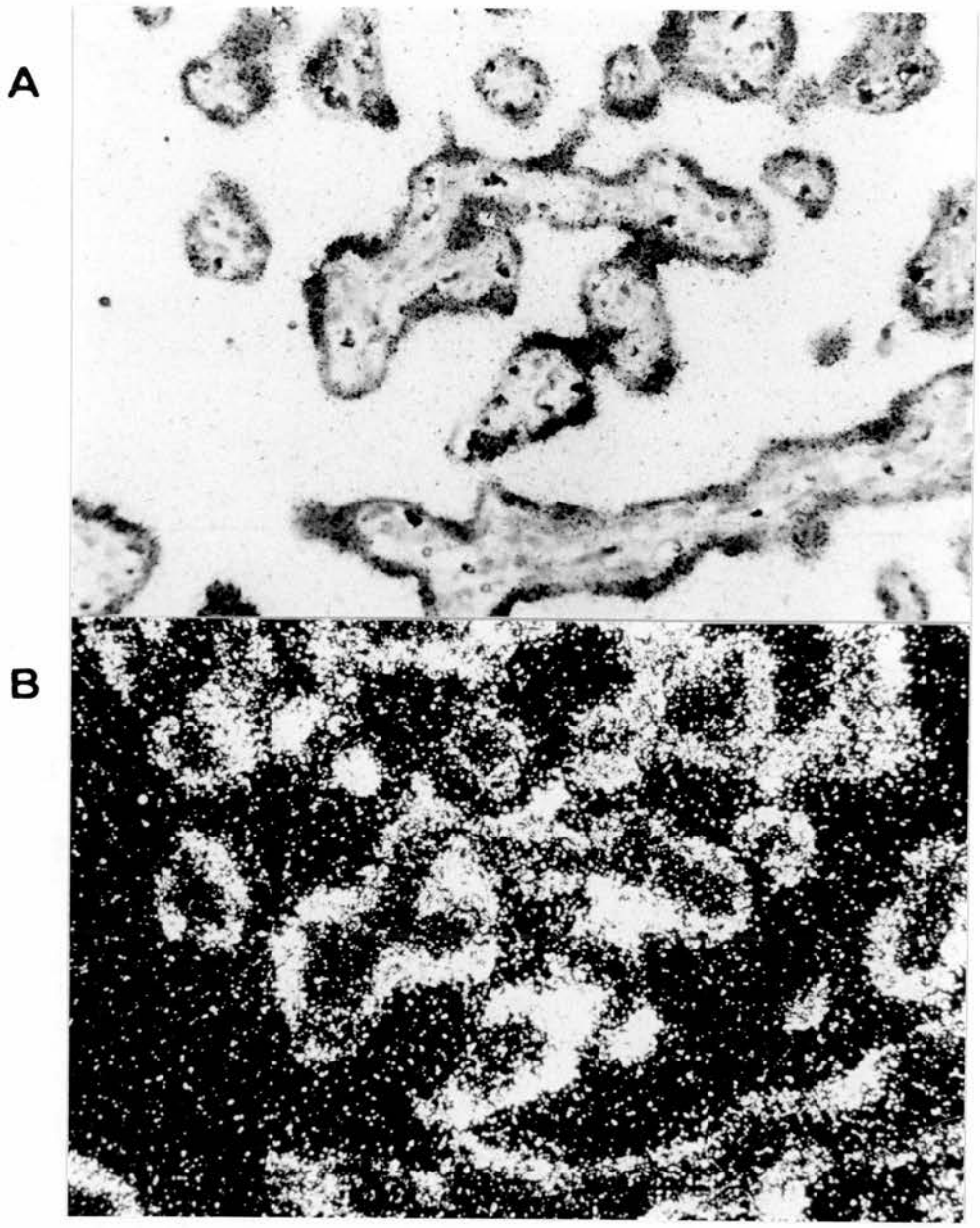

Figure 8.7 Hybridization of term placental tissue with inhibin βA-subunit riboprobe. Low-magnification (x20) bright field photomicrograph (A) and dark field photomicrograph (B) of the same area showing expression of inhibin βA-mRNA within trophoblast cells.

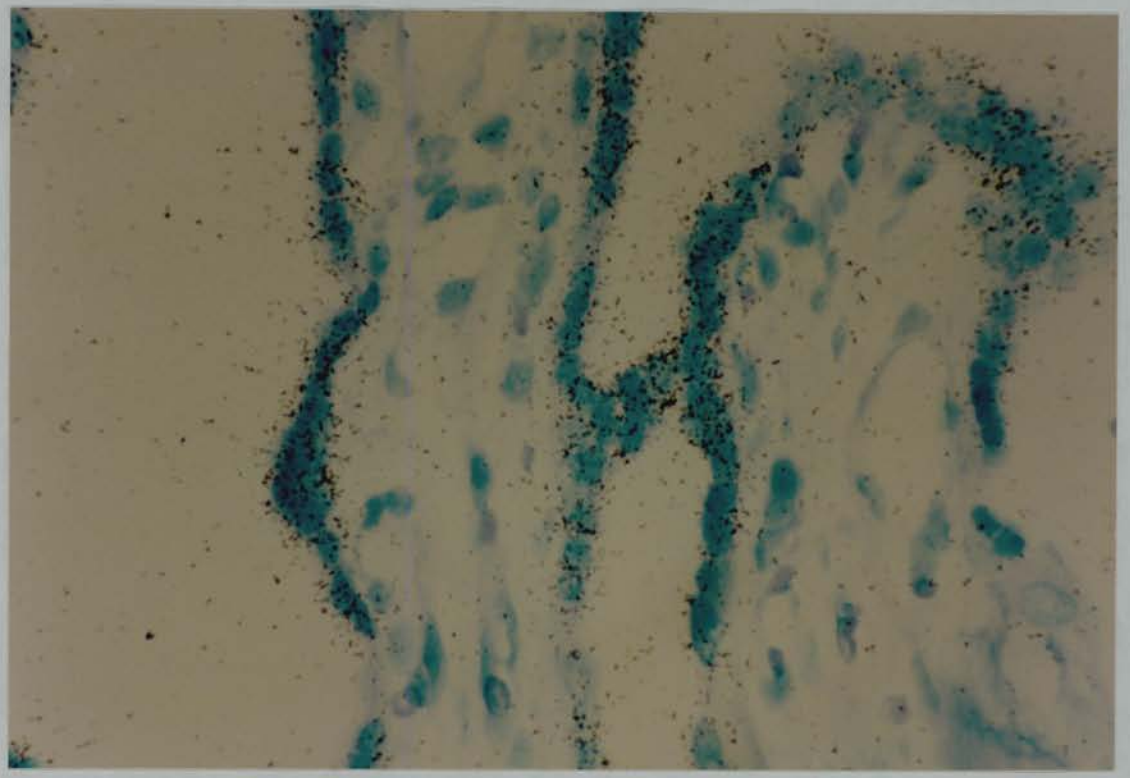

Figure 8.8 High-magnification photomicrograph (x40) demonstrating hybridization of the inhibin βA-subunit riboprobe in syncytiotrophoblast and cytotrophoblast cells of term placental tissue.

but the expression of this subunit mRNA (Figures 8.9 and 8.10) appeared low and a high background of non-specific signal was present. The same degree of hybridization was also demonstrated in term placenta and again the hybridization appeared in the area of the trophoblast cells (Figures 8.11 and 8.12). Comparing the hybridization of βB-antisense and βB-sense probes to 7 week and term placentae (Figures 8.13 and 8.14), the antisense probe clearly showed more specific hybridization signal.

8.3.2 Discussion

The data presented above demonstrate that inhibin α- and βA-subunit mRNAs are expressed in syncytiotrophoblast and cytotrophoblast cells of human chorionic villi throughout pregnancy. This finding, supported by the original observation of immunolocalization of inhibin α- and βA-subunit proteins in the same cell types (Tovanabutra et al, 1990; Petraglia et al, 1987a; Merchenthaler et al, 1987), has identified syncytiotrophoblast and cytotrophoblast cells as the site of inhibin production in the human placenta.

The amount of inhibin-subunit mRNAs detected in placental tissue sections varies during pregnancy. More inhibin α-subunit mRNA was present in early pregnancy than in late pregnancy and this confirms the earlier finding (Chapter 7) that inhibin α-subunit mRNA expression is decreased with age of gestation. Furthermore, high level of inhibin immunoactivity in placental extracts and strong intensity of α-inhibin subunit staining in early pregnancy are also consistent with this differential expression of the inhibin α-subunit gene.

Expression of inhibin βA-subunit mRNA occurs in early and term placentae but hybridization of the inhibin βB-subunit riboprobe did not show as strong a signal as the inhibin βA-subunit riboprobe. Although there is evidence of transient expression of inhibin βA- and βB-subunit genes in rat follicles during the oestrous cycle (Meunier et al, 1988a) and monkey follicles during growth and development (Schwall et al, 1990) this is unlikely to be the case in the human placenta. The pattern of increase in inhibin βA-subunit mRNA expression by trophoblast cells towards term is similar to the pattern of inhibin βB-subunit mRNA demonstrated by Northern blot analysis. This leads to the conclusion that inhibin βA- and βB-subunit genes are both expressed throughout pregnancy but the failure to demonstrate convincing expression of inhibin βB-subunit inhibin gene expression using the *in situ* hybridization technique may be due to the lack of a suitable riboprobe. This requires further investigation.

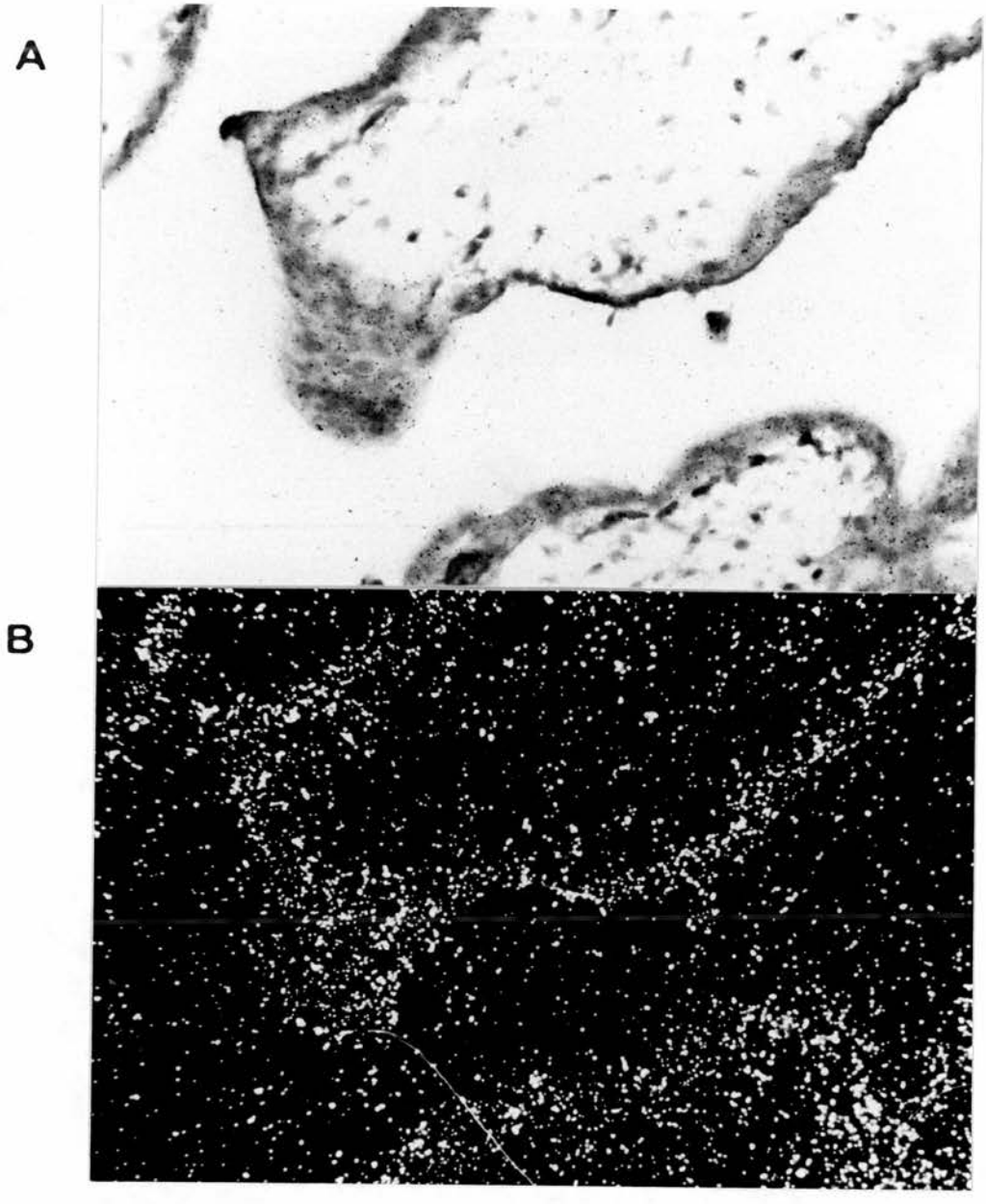

Figure 8.9 Hybridization of 7 week placental tissue with the inhibin βB-subunit riboprobe. Low-magnification (x20) bright field photomicrograph (A) and dark field photomicrograph (B) of the same area showing expression of inhibin βB-mRNA in the trophoblast layer.

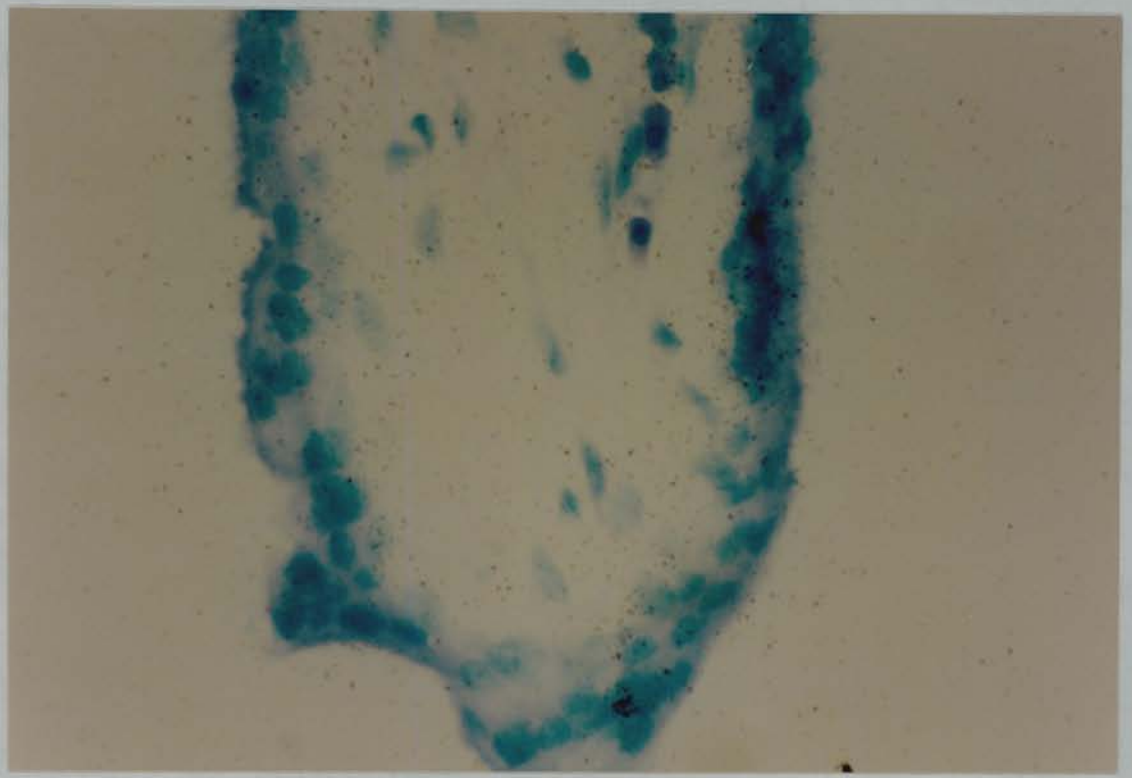

Figure 8.10 High-magnification photomicrograph (x40) showing hybridization of the inhibin βB-subunit riboprobe in syncytiotrophoblast and cytotrophoblast cells of 7 week placental tissue.

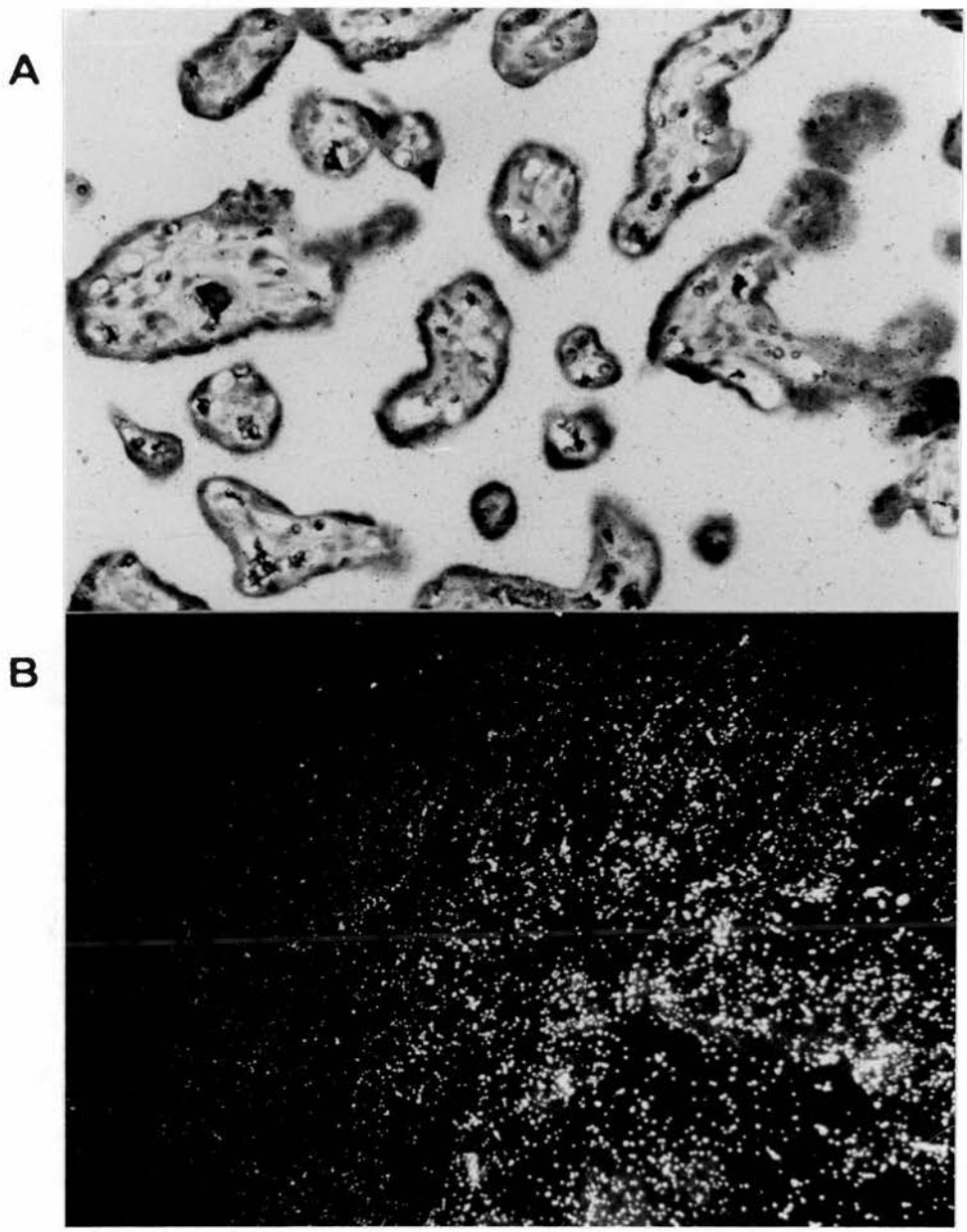

Figure 8.11 Hybridization of term placental tissue with the inhibin βB-subunit riboprobe. Low-magnification (x20) bright field photomicrograph (A) and dark field photomicrograph (B) of the same area showing slight expression of inhibin βB-mRNA in chorionic villi.

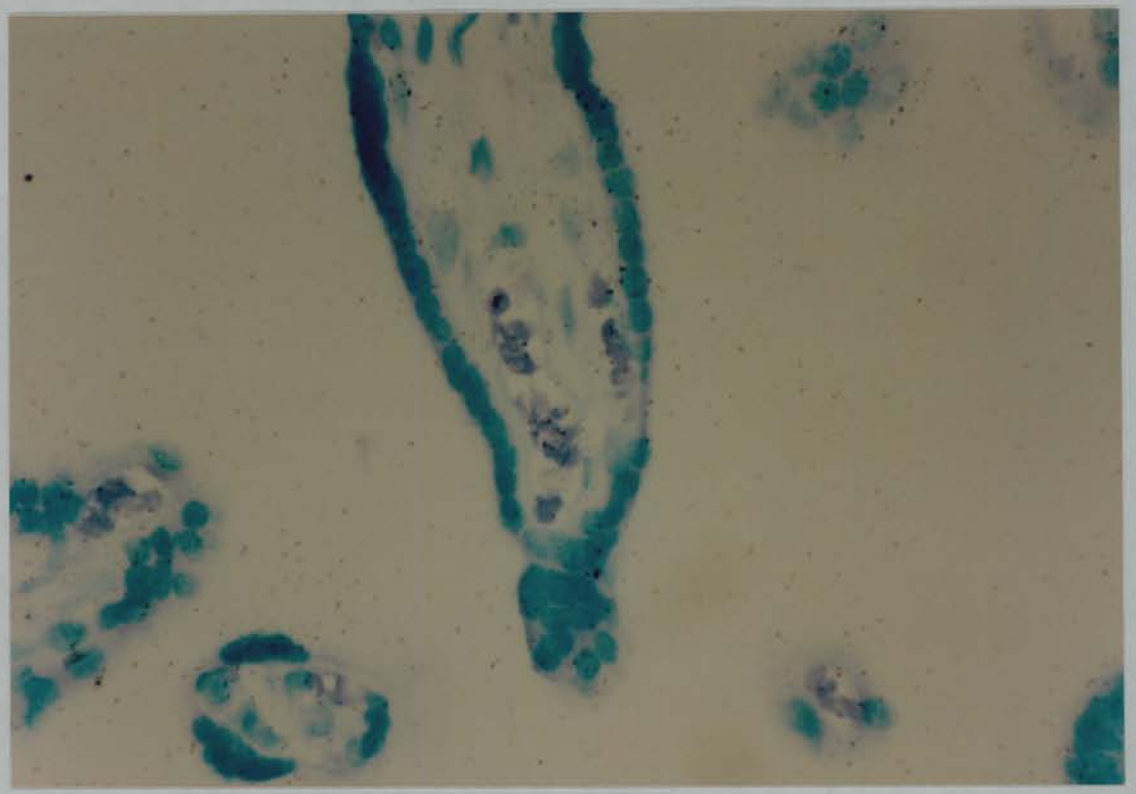

Figure 8.12 High-magnification photomicrograph (x40) showing hybridization of the inhibin βB-subunit riboprobe in term placental tissue.

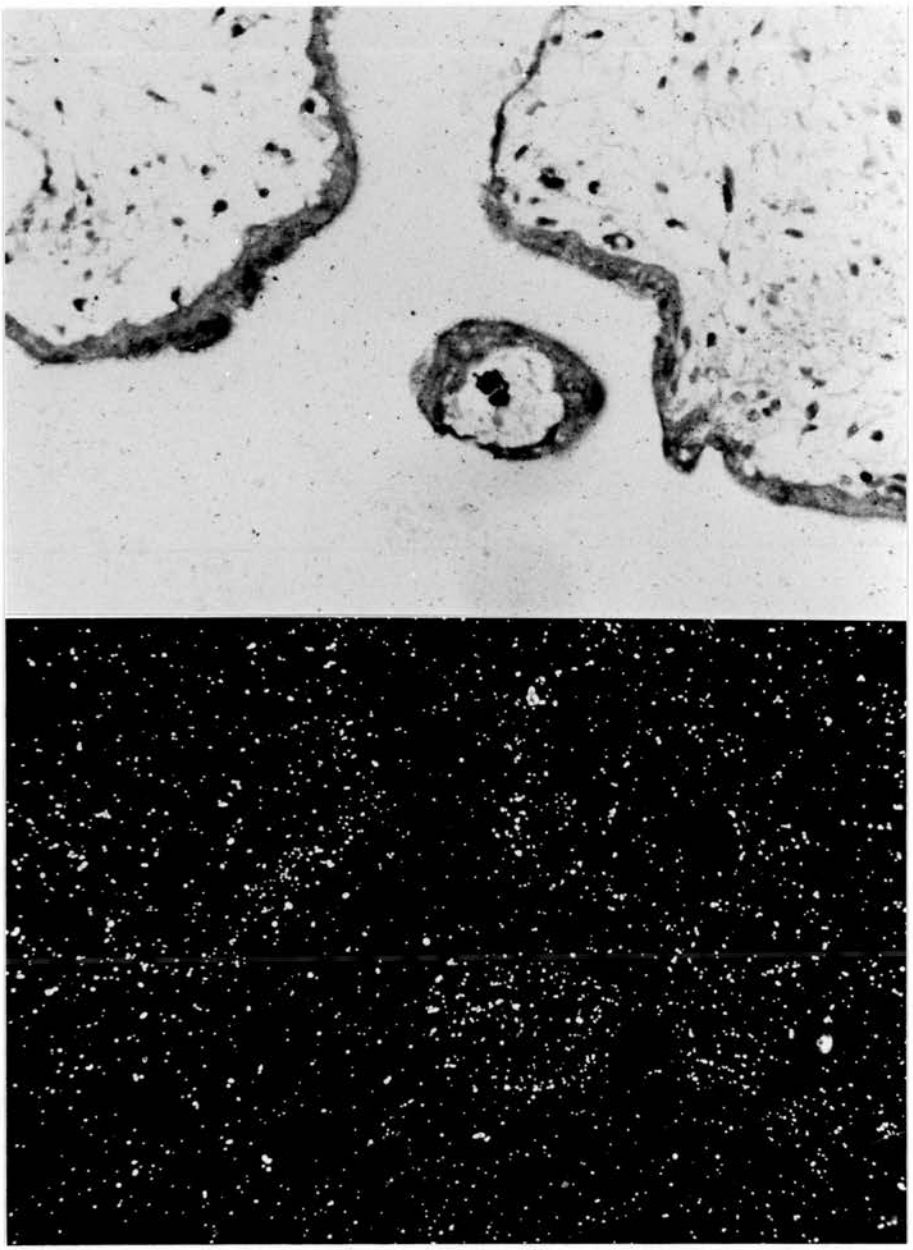

Figure 8.13 Photomicrograph showing no hybridization of sense inhibin βB-riboprobe to 7 weeks placenta.

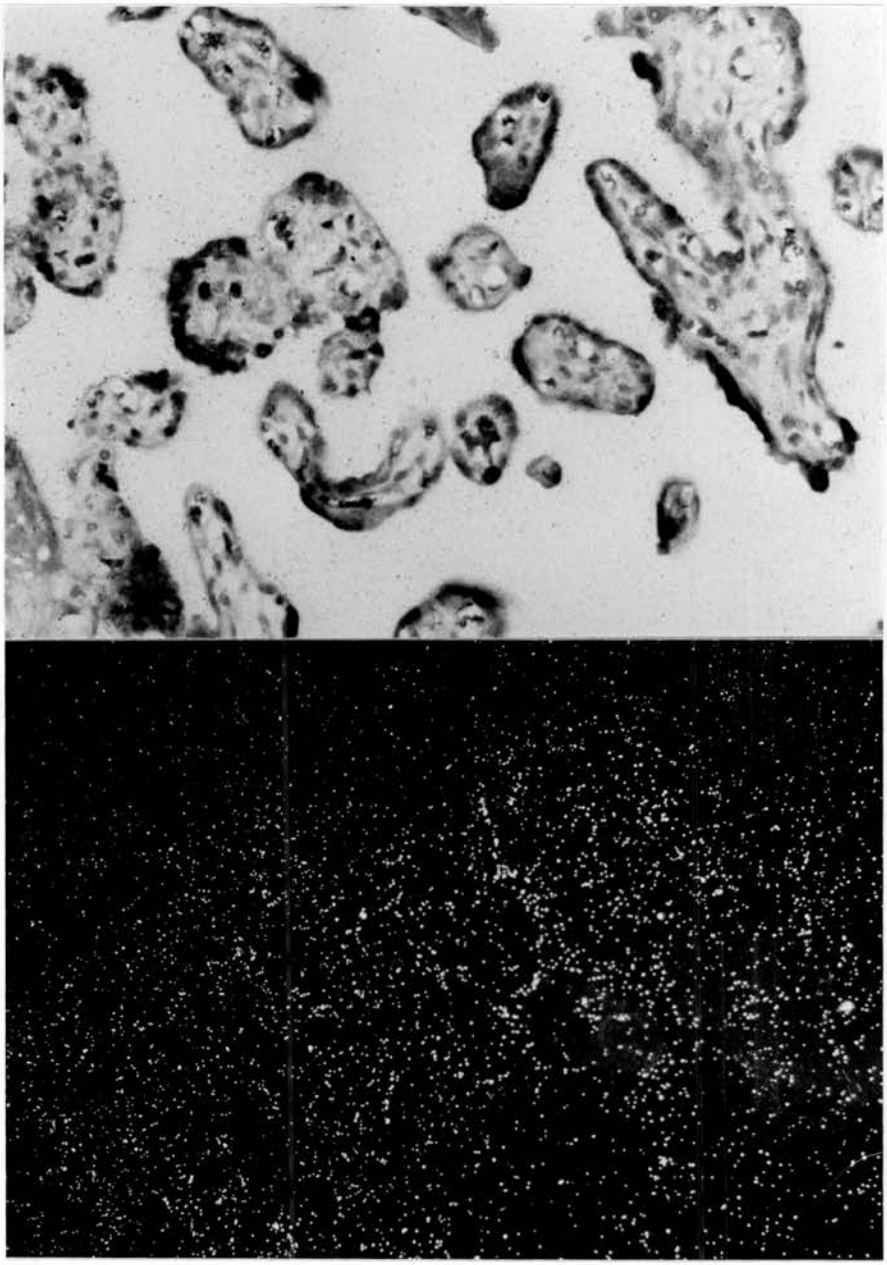

Figure 8.14 Photomicrograph showing no hybridization of sense inhibin βB-riboprobe to term placenta.

There have been previous reports on the preferential production of inhibin βA-subunit dimers by the human leukaemia cell line THP-1 (Eto et al, 1987) as well as in bone marrow where the expression of inhibin βA-subunit mRNA is the predominant form (Meunier et al, 1988b). In addition, Mason (1988) has demonstrated that in a mammalian cell line transfected with inhibin cDNAs, inhibin heterodimers are only produced in the presence of an excess of inhibin α-subunit gene whereas inhibin β-monodimers are preferentially formed when there are equimolar ratios of α- and β-subunit proteins.

This current study suggests that in placentae the expression of the inhibin α-subunit gene decreases while the expression of the inhibin βA-subunit gene increases with the age of gestation. This provides further evidence for the hypothesis that production of activin by trophoblast cells is favoured in late pregnancy. This has been confirmed by our previous findings showing the *in vitro* stimulatory effect of activin-like substance in mid-term and term placental extracts.

In conclusion, the results from this study clearly show that cytotrophoblast and syncytiotrophoblast cells are the sites of inhibin α- and β-subunit protein production in the human placenta and the amount and species of secreted proteins vary during pregnancy. Moreover, this finding also supports the concept proposed in Chapter 6 on a paracrine and autocrine role for inhibin within trophoblast cells.

CHAPTER 9

General Discussion
Inhibin in pregnancy

Among the various tissues and organs in the human body, the human placenta was reported to possess bioactive and immunoactive inhibin (Bandivdekar et al, 1981; McLachlan et al, 1986b). The presence of inhibin in a totipotential tissue such as placenta, which has an important role and complex functions in supporting the growth and development of the fetus throughout pregnancy, raises questions about the function of inhibin. In the past five years, there has been much information obtained by different investigators on the synthesis and role of inhibin present in human term placentae. Nevertheless, many more studies are required to understand the role of inhibin in pregnancy. The studies described in this thesis provide further information on the molecular species of inhibin and its related peptides produced by the human placenta, the potential sources of peripheral inhibin during pregnancy, the cellular localization of inhibin subunit proteins including the expression of mRNA and the presence of inhibin in amniotic fluid.

The molecular species of inhibin produced by the placenta and the relative amounts during gestation were examined in Chapter 2. Placental extracts from various stages of pregnancy showed parallel displacement with hFF in the radioimmunoassay with the Monash and Y29 antibodies whereas the results from Chapter 4 also demonstrated parallelism between dose-response lines of placental extracts and hFF during the measurement of bioactive inhibin using the sheep pituitary cell monolayer culture. These findings suggest that the same species of immunoactive and bioactive inhibin is secreted by the ovary and placenta during pregnancy.

The level of inhibin immunoactivity in human placenta is highest in the early weeks of gestation (7 weeks) and declines significantly towards term whereas the bioactivity remains constant throughout pregnancy. Further investigations in Chapter 4 on inhibin bioactivity in placental extracts by *in vitro* immunoneutralization using anti-inhibin α-subunit antibody (Y29) confirms that the inhibin-like bioactivity in placental extract is due to dimeric inhibin produced by the placenta. The results from the same experiment also provide the indirect evidence for the presence of activin-like activity in the human placenta from the late stages of pregnancy. When inhibin bioactivity in mid- and term

placental extracts was completely immunoneutralized by antibody (Y29), the release of FSH was stimulated above the level found in control pituitary cell cultures. In addition, the observed FSH stimulatory effect of placental extracts is similar to that shown by recombinant activin A in Chapter 4. This suggests that activin is produced by the placenta in the second and third trimesters. However, the RIA with the Monash antibody has been shown to measure pro-αC which is found in abundance in hFF and has no bioactivity whatsoever. Thus, changes in the ratio of immunoactivity to bioactivity might be due to different molecular species of inhibin being produced during pregnancy.

It was previously reported that the placenta was the only source of inhibin in pregnancy due to the finding that inhibin levels increased within a few days of implantation in pregnant women who had undergone a superovulation induction and follicular aspiration for the purpose of *in vitro* fertilization (McLachlan et al, 1987c) and in agonadal women who became pregnant following embryo donation (McLachlan et al, 1987b). In Chapter 3, the pattern of peripheral inhibin levels in pregnancy along with progesterone and hCG from day 0 of pregnancy (day of the LH peak) until day 65 provide strong evidence that in the early stages of pregnancy the corpus luteum is the main source of inhibin. The significant correlation between hCG and inhibin levels including their pattern of secretion during early pregnancy, suggests a relationship between hCG and inhibin production by the corpus luteum and it is clearly shown that hCG at concentrations similar to those seen in early pregnancy can stimulate inhibin secretion by the corpus luteum in normal women (Illingworth et al, 1990).

An increase in inhibin levels was observed within a few days after implantation (McLachlan et al, 1987c), but the pattern of inhibin secretion in that study might be unusual because of the result from superovulation induction. However, the presence of inhibin immunoactivity in placental extracts provides strong evidence that from 7 weeks of pregnancy until term, the placenta is another source of inhibin. The pattern of inhibin immunoactivity in plasma differs from that shown in placental extracts. The rise in peripheral inhibin levels throughout pregnancy as shown in Chapter 3 and also by Abe et al (1990) and Tabei et al (1991) is in contrast to the decline in the concentration of inhibin in placentae towards term (Chapter 3). This is presumably due to an increase in placental weight and blood flow. This presumption also applies to the different patterns of bioactive inhibin in the circulation and in placenta extracts. The concentration of bioactive inhibin in peripheral plasma measured using the sheep pituitary cell bioassay rises progressively (Qu et al, 1991) whereas in the placenta it

remains the same throughout pregnancy (Tovanabutra et al, 1990). Interestingly, the patterns of inhibin and oestradiol are very similar. The incompatibility between the pattern of peripheral immunoactive and bioactive inhibin may be explained by different inhibin species being produced at various stages of pregnancy. It should be remembered that steroids e.g. oestradiol and progesterone also have an FSH-suppressing effect in sheep pituitary cell cultures. Thus it is important to ensure that all residual steroids are removed from the sample before a reliable assessment of inhibin bioactivity can be obtained using the sheep pituitary cell bioassay.

Inhibin (α-, βA- and βB-) subunit mRNA is expressed in human term placenta (Mayo et al, 1986; Davis et al, 1987; Reddi et al, 1990b) as demonstrated by Northern blot analysis. These findings provide conclusive evidence that inhibin subunits are synthesized within term placental tissues. The expression of inhibin α- and β-subunit mRNAs in placental tissue from 7 weeks, 16 weeks and term, further demonstrate the synthesis of inhibin subunits within the placenta throughout pregnancy (Chapter 7). The expression of these subunits varies during gestation. The high degree of expression of α-subunit mRNA at 7 weeks of pregnancy suggests preferential production of the free α-subunit of inhibin and the higher expression of β-subunit mRNA in second trimester and term suggests the production of activin which is compatible with the activin-like activity in mid- and term placental extracts demonstrated in Chapter 4.

It is known that cytotrophoblast cells contain the inhibin α-subunit (Petraglia et al, 1987a; Merchenthaler et al, 1987). The studies in Chapter 3 show that both inhibin α- and βA-subunits are localized in cytotrophoblast and syncytiotrophoblast cells throughout pregnancy. This result is in agreement with the presence of bioactive inhibin in placental extracts. The difference in intensity of staining at various stages of pregnancy seems to suggest a differential production of inhibin subunits. The high intensity of α-subunit staining in 7 weeks placental tissues and an increase in intensity of inhibin βA-subunit staining again suggest the preferential production of the free α-subunit or pro α-C when compared to dimeric inhibin, and the production of activin in later stages of pregnancy. It can be stated that there are many factors which could influence the intensity of staining apart from the amount of immunoactive proteins. However, with the evidence from other studies (e.g. gene expression, immunoactivity and immunoneutralization) it is tempting to conclude that under these circumstances the intensity of staining is proportional to the amount of inhibin subunit proteins.

The last finding of this thesis is the localization of inhibin α- and βA-subunit mRNAs in trophoblast cells using *in situ* hybridization. The degree of expression of inhibin α- and βA-subunit mRNAs in 7 weeks and term placental tissues are different but are in keeping with the results from the immunolocalization study which shows that, in the early stages of pregnancy, trophoblast cells produce more α-subunit than at term and more β-subunit is produced as the pregnancy progresses. This is very conclusive evidence which reveals that most inhibin and/or activin secreted by the placenta is synthesized by trophoblast cells.

The presence of inhibin subunits in decidual tissue has recently been demonstrated by immunohistochemistry using affinity-purified polyclonal rabbit antibodies raised against N-terminal synthetic porcine peptides of inhibin α-, βA- and βB-subunits (Petraglia et al, 1990). These authors also demonstrated different degrees of gene expression of these subunits at various stages of pregnancy by Northern blot analysis. However, there is a discrepancy between the small amounts of inhibin βA-subunit protein detected by immunocytochemistry and its high degree of gene expression detected by Northern blot analysis in term decidual tissue. Taken together with the fact that the decidua contains migratory cells of trophoblast origin and our observation that the cultured medium from decidua parietalis contains very little immunoactive inhibin whereas medium from decidua capsularis contains a considerably greater amount, it is not entirely clear whether maternally-derived decidual cells can produce inhibin, and this needs further investigation.

There is evidence for the presence of inhibin in mid-term amniotic fluid (Sinosich et al, 1991) and activin A in amniotic fluid (Abe et al, 1989). The study in Chapter 5 demonstrates both inhibin immunoactivity and bioactivity in amniotic fluid throughout pregnancy. The source of inhibin in amniotic fluid remains unclear. It is likely that trophoblast and decidual tissues and possibly the fetus are potential sources of inhibin and activin in amniotic fluid. The circulating inhibin in umbilical cord is shown to be lower than in maternal blood vessels and there is no difference between umbilical vein and artery levels (McLachlan et al, 1986b; Tabei et al 1991; Kettel et al, 1991) indicating that the fetus is not contributing to the peripheral immunoactive inhibin. However, the results in Chapter 5 demonstrate the presence of inhibin bioactivity and immunoactivity in 7 week amniotic fluid, suggesting the production of inhibin by the fetus or trophoblast.

The physiological function of inhibin and activin in pregnancy remains unknown. In a normal woman, the endocrine function of inhibin is likely to be a synergistic effect with oestradiol on suppressing FSH secretion. The concentration of immunoactive FSH in the circulation is very low during pregnancy (Jaffe et al, 1969) which suggests a combined suppressive effect of inhibin and oestradiol on the secretion of pituitary FSH and this hypothesis is supported by the evidence that throughout pregnancy the development of antral follicles continues although they never achieve the final stages of maturation beyond 10 mm (Govan, 1968). Several studies have reported relatively high levels of bioactive FSH in serum samples in early pregnancy (Padmanabhan et al, 1989). It may be that this bioactivity represents an intrinsic property of hCG which has both LH and FSH activity.

A paracrine function of inhibin and activin in pregnancy with other hormones was proposed by Petraglia et al, (1987a; 1989). In placental cell cultures, activin has no effect on hCG secretion but it can augment the stimulation by GnRH of hCG release as well as stimulate progesterone secretion. These effects of activin can be reversed by inhibin (Petraglia et al, 1989). Inhibin also suppresses the stimulatory effects of GnRH on hCG secretion (Petraglia et al, 1987a). Interestingly, in placental explant tissue culture inhibin suppresses hCG secretion by term placenta but not by first trimester placenta (Mersol-Barg et al, 1990). This suggests a transient interaction of inhibin and hCG during pregnancy. However, our finding in Chapter 6 that inhibin α- and βA-subunits are present in the same cell type strongly suggests an autocrine function between inhibin, activin and hCG.

Structural analysis of inhibin shows homology between inhibin and other growth factors e.g TGF-β, MIS, activin, the VG1 gene in *Xenopus* and decapentaplegic peptides in *Drosophila*, all of which are growth factors needed for embryogenesis in different species (see de Kretser & Robertson, 1989). Taken together with the results in Chapter 3 that in early pregnancy the corpus luteum secreted large amounts of inhibin, it is likely that inhibin has a potential role in the early development of the embryo or in the establishment of pregnancy.

Since inhibin is produced by trophoblast cells, it may be a potential marker of trophoblastic disease in women. A single study suggests that, apart from hCG, inhibin may be another circulating marker of hydatidiform mole and even better than hCG as an early indicator of persistent trophoblastic disease (Yohkaichiya et al, 1989). Hydatidiform mole is due to cystic degeneration of the chorionic villi; the trophoblast

cells persist and invade while the mesodermal core undergoes myxomatous change and blood vessels disappear. The incidence of hydatidiform mole is 1 in 200 in Asian and African countries – which is 10 times higher than in Western countries. About 10% of hydatidiform moles become malignant and give rise to choriocarcinoma. This disease is now curable with chemotherapy if the treatment is started early enough and this means that an effective diagnostic marker is needed.

It would be worthwhile to conduct a study to investigate the relationship between inhibin and other reproductive hormones and some growth factors in normal pregnancy and trophoblastic diseases using the placental explant culture as an experimental model. This would enable us to understand more about the physiological role of inhibin and elucidate suitable and effective markers for the diagnosis of these trophoblastic diseases.

Appendices

Appendix 1

1.1 Phosphate-buffered saline (PBS)

Dissolve 1 tablet of phosphate-buffered saline (Oxoid, Unpath Ltd, Basingstoke, Hampshire, England, Code BR 14a) in 100 ml distilled water and autoclave at 115°C for 10 min and adjust to pH 7.4 with 5 N NaOH.

1.2 4% paraformaldehyde in PBS, pH 7.4

Dissolve 4 g of paraformaldehyde (Aldrich Chemical Company Ltd., Gillingham, Dorset) in 100 ml of PBS at 60°C. Add a few drops of 5 N NaOH to completely dissolve the paraformaldehyde and adjust the pH of the solution to 7.4 with conc. HCl.

Appendix 2

2.1 Denaturing solution

A stock solution was prepared by dissolving 250 g of guanidinium thiocyanate (Fluka) in the manufacturer's bottle with 293 ml sterile water. Add 17.6 ml of 0.75 M sodium citrate, pH 7.0 and 26.4 ml of 10% sarcosyl (N-laurylsarcosine, Sigma). Incubate at 65°C until the solution is clear. The shelf-life of this solution is 3 months at room temperature.

Prepare the final denaturing solution by adding 0.36 ml of 2-mercaptoethanol (Sigma) to 50 ml of the stock solution.

2.2 Water- saturated phenol.

100 g phenol (Gibco-BRL) was melted at 68°C. Sterile water was added up to the neck of the bottle and the mixture was left to separate at 4°C overnight. Most of the water was removed and the solution stored at 4°C for up to 1 month.

2.3 Determination of the amount and purity of RNA

To estimate the amount and purity of RNA, 4 µl of RNA obtained from the extraction step was made up to 1 ml with sterile water. The solution is spectrophotometrically read at 260 nm and 280 nm respectively. An optical density (OD) reading of 1 at 260 nm corresponds to approximately 40 µg/ml of RNA which allows an estimation of the total concentration of RNA in the preparation after correcting for dilution. The ratio

between the readings at 260 nm and 280 nm provides an estimation as to the purity of the RNA. Pure preparations give an OD260/OD280 value between 1.8-2.0.

2.4 Loading buffer

Mix 200 µl 10x running buffer, 1ml deionized formamide and 356 µl formaldehyde (37% solution, Sigma).

2.5 Deionized formamide

Mix 5 g of a mixed-bed resin (Amberlite MB-1, Sigma) with 50 ml of formamide (Sigma). Stir gently at 4°C overnight and filter through Whatman No.1 filter paper. Store at -20°C.

2.6 10% formaldehyde agarose gel running buffer.

Dissolve 8.36 g of 3-(N-morpholino) propanesulfonic acid (MOPS, Sigma), 0.744 g EDTA and 0.82 g of sodium acetate in 150 ml of sterile water. Adjust the pH of the solution to 7.0 with RNAse-free 5 M NaOH and bring the final volume to 200 ml with sterile water.

2.7 Casting of 0.8% agarose gel

Dissolve 1.5 g of SeaKem ME agarose gel (ICN) in 72.1 ml of sterile water.

Heat the solution in a microwave oven at 100% heat for 3 min or until the agarose dissolves.

Cool the solution to 60° C and add 17.9 ml of formaldehyde (37% solution, Sigma) with gentle swirling.

Pour the gel on to a prepared mold (20x15 cm) with a 15 well comb and let the gel set at room temperature (20-30 min). Remove the comb and mount the gel in the electrophoresis tank containing 1.5 litre of 1x running buffer.

2.8 20X SSC

Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 ml DEPC-treated water. Adjust the pH of the solution to 7.0 with 10 N NaOH and bring the volume up to 1 litre with DEPC-treated water.

2.9 Buffer-saturated phenol

Add 8-hydroxyquinone (Sigma) to water-saturated phenol at a final concentration of 0.1%. Add an equal volume of 1.0 M Tris buffer (pH 8.0). Stir on a magnetic stirrer for 15 min and discard the buffer layer. Repeat the extractions with an equal volume of

0.1M Tris buffer (pH 8.0) until the pH of the phenol phase is less than 7.8 and then store under the same buffer for up to 1 month.

2.10 Phenol:Chloroform (1:1)

An equal volume of buffer-saturated phenol and chloroform was mixed well.

Appendix 3

3.1 Preparation of tespa-coated slides.

Dip the pre-washed and pre-baked new glass slides in the following solutions in order:
10% HCl in 70% EtOH (10 sec).
Sterile water (10 sec).
95% EtOH (10 sec).
Dry the slides at 150°C for 5 min and allow to cool.
2% tespa (3-aminopropyltriethoxy-silane, Sigma) in acetone (BDH) for 10 sec.
100% acetone twice (10 sec each).
Sterile water (10 sec).
Dry the slides at 42°C overnight.

3.2 Preparation of Terrific Broth

In a microwave oven, dissolve 12 g bactotryptone (Gibco), 24 g yeast extract (Gibco) and 4 ml glycerol (Sigma) in 900 ml of sterile water and then autoclave to sterilize. Allow the solution to cool and then add 5 ml of sterile phosphate buffer solution containing 0.17 M KH_2PO4 (BDH) and 0.72 M K_2HPO4 (BDH) to 45 ml of the solution.

3.3 Plasmid purification

Prepare a spun column as follows:
Invert the column several times to suspend the Sephacryl S-400 gel.
Set the column upright in a rack and allow the gel to settle into a continuous bed.
Remove the top cap and then the bottom closure. Allow the column to drain by gravity but do not let it dry. Wash the column with 2 ml of 0.1 M triethanolamine, pH 8.0 (TE buffer) twice.
Place the column in a 15 ml polypropylene, round-bottomed tube (Falcon) and spin at 400g for 2 min. The gel will appear compacted and, at the top, will have retracted slightly from the inside wall of the column.
Remove the column from the tube and discard the eluate.

Place a microcentrifuge tube in the bottom of the polypropylene tube and place the column in the same tube. The tip of the column must be inside the microcentrifuge tube. The column is now ready for the application of the sample.

3.4 DNA estimation

A rapid way to estimate the amount of DNA in the effluent from the spun column is to utilize the UV- induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, when the fluorescent yield of the unknown is compared to that of a series of standards (0.5-20 µg/ml or ng/µl) small amounts of DNA can be detected.

Procedure:

Cut a piece of 3" x 4" parafilm and place on a small plastic tray.

Spot 0.5 µl of DNA sample on to the parafilm sheet and add 4.5 µl of sterile water to the DNA spot.

Spot 5 µl of standards by arranging them in series.

Add 5 µl of ethidium bromide solution (2 µg/ml of ethidium bromide in TE buffer, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and mix well by pipetting.

Photograph the spots under UV light. Estimate the concentration of DNA by comparing the intensity of fluorescence in the sample with the standard

3.5 10x "Orange Juice" (gel loading buffer for DNA)

0.25% Orange G (Sigma O 7252)

15% Ficoll (Type 400, Pharmacia)

0.5 M EDTA

Make the solution up in 10x Tris-borate buffer (TBE, 0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA) and store at -20°C

3.6 Casting of 0.8% agarose gel

Add 0.4 g of SeaKem agarose gel (ICN) to 50 ml TBE in a conical flask.

Heat the solution in a microwave oven at 50% heat for 3 min or until the agarose dissolves.

Cool the solution to 50° C and add 2.5 µl of ethidium bromide (10 mg/ml) to give a final concentration of 0.5 µg/ml.

Pour the gel on to a prepared mold. Immediately place the comb and let the gel set at room temperature (20-30 min). Remove the comb and mount the gel in the electrophoresis tank with 200 ml of TBE buffer as the running buffer.

3.7 DEPC-treated water

Make the stock DEPC by diluting 5 ml of diethylpyrocarbonate (DEPC; Sigma) in 45 ml EtOH. Dispense 500 µl of stock solution into a prebaked bottle, dry down EtOH under N_2 and add 500 ml distilled water. Leave the solution (0.01% DEPC) to stand overnight and then autoclave.

3.8 Hybridization mix

Make up a solution containing the following:
50% deionized formamide (Sigma)
10% Dextran sulphate (Sigma)
1x Denhardt's solution (Sigma)
20 mM Tris, pH 8.0
0.3 M NaCl
5 mM EDTA
10 mM sodium phosphate
0.5 mg/ml yeast RNA (Sigma)
50 mM dithiothreitol (add immediately before use)

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